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Neutral Polysaccharide from *Bletilla striata* (Thunb. ex A. Murray) Rchb. f. Alleviates Liver Fibrosis through Modulation of TGF- β /SMAD Signaling Axis and Remodeling of Gut Microbiota

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ABSTRACT: Hepatic fibrosis remains a global health concern that urgently requires development of new anti-fibrotic compounds with high efficacy and low toxicity. *Bletilla striata*, known for its high polysaccharide content and well-established safety profile, shows therapeutic potential. In this study, we isolated a novel neutral glucomannan (BsPsIII1-1, approximately 74.8 kDa) from *B. striata*. Structural analysis showed that the proposed main chain repeating unit was $\rightarrow\{4\}\text{-}\beta\text{-D-Glcp-(1)}_x\rightarrow\{4\}\text{-}\beta\text{-D-Manp-(1)}_n\rightarrow\{4\}\text{-}\beta\text{-D-Glcp-(1)}_y\rightarrow 4\text{-}\beta\text{-D-2Ac-Manp-(1}\rightarrow 4\text{)-}\beta\text{-D-3Ac-Manp-(1}\rightarrow 4\text{)-}\beta\text{-D-2Ac-Manp-(1}\rightarrow 4\text{)-}\beta\text{-D-Manp-(1)}_m\rightarrow 4\text{)-}\beta\text{-D-3Ac-Manp-(1}\rightarrow 4\text{)-}\beta\text{-D-Manp-(1)}_i\rightarrow$ (where $x+y=8$ and $n+m+i=4$). BsPsIII1-1 attenuated hepatic fibrosis both *in vitro*, by reducing TGF- β 1-induced α -smooth muscle actin (α -SMA) and collagen type I (COL-1) in LX2 cells, and *in vivo*, by mitigating CCl₄-induced weight loss, AST and ALT elevation, and hepatic MDA and GSH imbalance in mice, with histopathology confirming decreased collagen deposition. Mechanistically, BsPsIII1-1 inhibited TGF- β /SMAD signaling by inhibiting TGF- β 1 secretion, suppressing phosphorylation of SMAD2 and SMAD4, and increasing SMAD7. It also modulated gut microbiota by enhancing beneficial bacteria, suppressing pathogens, and promoting production of short-chain fatty acids. These actions synergistically downregulated α -SMA and COL-1 through the gut-liver axis and TGF- β /SMAD signaling. In our study, we identified BsPsIII1-1, a novel biomacromolecule isolated from *B. striata*, as a potential anti-fibrotic agent with hepatoprotective properties, demonstrating promise as a functional food or therapeutic candidate for the treatment of fibrotic liver diseases.

Keywords: *Bletilla striata* polysaccharide; structural characterization; hepatic fibrosis; TGF- β /SMAD signaling pathway; gut microbiota

1. Introduction

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Hepatic fibrosis, a common pathological process in chronic liver diseases, is characterized by excessive deposition and aberrant distribution of extracellular matrix (ECM) components, including collagen, glycoproteins, and proteoglycans^[1]. The development of liver fibrosis is commonly accompanied by multiple chronic liver diseases, including viral hepatitis, drug-induced liver injury, alcoholic or nonalcoholic fatty liver disease (NAFLD), and autoimmune liver diseases^[2, 3]. Chronic hepatic fibrosis results in progressive structural and functional deterioration of the liver, significantly worsening the prognosis of chronic liver diseases and increasing the risk of severe complications, including cirrhosis and hepatocellular carcinoma^[1].

As both a response to persistent hepatic injury and a critical determinant of long-term clinical outcomes^[4], hepatic fibrosis represents a key therapeutic target. Although early intervention can reverse hepatic fibrosis at the histological level, its progression to cirrhosis or hepatocellular carcinoma markedly reduces reversibility. Moreover, these advanced liver pathologies not only increase treatment costs but also impose substantial psychological burdens on patients and their families^[5]. Therefore, pharmacological reversal of hepatic fibrosis represents a critical clinical objective in chronic liver disease management. Nevertheless, existing antifibrotic agents remain largely experimental, with no approved specific therapies for hepatic fibrosis^[6]. Furthermore, many existing antifibrotic compounds exhibit toxicity that limits their therapeutic efficacy^[7], leaving an urgent unmet clinical demand for safe and effective drugs capable of halting or reversing fibrosis progression.

Various polysaccharides have demonstrated therapeutic potential in fibrosis treatment. For example, polysaccharides from *Astragalus membranaceus* enhanced hepatocyte antioxidant capacity, promoted tissue regeneration, mitigated cellular damage, suppressed hepatic stellate cells (HSCs) activation and proliferation, and modulated immune function against fibrosis^[8, 9]. Similarly, polysaccharides from *Dendrobium loddigesii* Rolfe (DLPs), exerted hepatoprotective effects by ameliorating liver injury and reducing inflammatory necrosis in hepatocytes through modulation of lipid metabolism, the gut-liver axis, and gut microbiota diversity^[10]. Some polysaccharides have even progressed to clinical trials. For example, belapectin, a galectin-3-inhibiting polysaccharide, has demonstrated efficacy in mitigating hepatic fibrosis and portal hypertension in preclinical models (mice) and exhibited safety in Phase 1 trials, leading to FDA Fast Track designation for nonalcoholic steatohepatitis (NASH) and cirrhosis clinical development^[11]. These discoveries have generated increasing interest in polysaccharides as antifibrotic agents.

Our study focused on *Bletilla striata*, a famous plant resource in China. This species is renowned for its established role in traditional medicine^[12] and has promising potential as a functional food^[13]. Its historical applications included lung nourishment, hemostasis, swelling reduction, tissue regeneration, and wound healing^[8, 14]. In terms of structure, polysaccharides from *B. striata* (BsPs) are the principal bioactive constituents, characterized as neutral glucomannans composed of mannose (Man) and glucose (Glc) in specific proportions linked by β -1,4-glycosidic bonds^[12, 15, 16]. Some BsPs may contain minor monosaccharides such as galactose (Gal)^[17], as evidenced in our prior work detecting Gal and arabinose (Ara)^[12]. The core structure of BsPs is predominantly composed of 1,4-linked Man and Glc residues. Although BsP

structures were first characterized in 1973, ongoing investigations continue to elucidate their structural diversity [14, 16, 18].

In terms of biological activity, BsPs exhibit broad anti-fibrotic potential across multiple organ systems. For example, a 50.06 kDa glucomannan with a Glc: Man ratio of 3.1:6.9 inhibited fibrosis in L929 cells by downregulating TGF- β 1, collagen type I (COL-1), vimentin, and α -smooth muscle actin (α -SMA), while also ameliorating pulmonary fibrosis in mice via p62 and *p*-mTOR modulation [19]. Similarly, a 260 kDa glucomannan known as BSPb with a Glc: Man ratio of 1:3 suppresses fibrosis in human mesangial cells by targeting TGF- β RI/II and α -SMA [20]. Further mechanistic studies revealed that BSPb reduced reactive oxygen species (ROS), IL-6, and TNF- α *in vitro*, inhibited NADPH oxidase 4 (NOX4) upregulation, and reduced the Ang II-induced toll-like receptor 2 (TLR2) overexpression, thereby exerting anti-renal fibrosis effects [21]. Emerging evidence suggests that BsPs exhibit anti-hepatic fibrosis properties. Low-molecular-weight BsPs have been shown to attenuate carbon tetrachloride-(CCl₄) induced hepatic fibrosis via the TLR2/TLR4-MyD88-NF- κ B signaling pathway [22]. Additionally, oligosaccharides derived from *B. striata* have demonstrated efficacy in ameliorating NAFLD [23]. Given their nontoxicity, excellent biocompatibility, strong biodegradability, abundance, and low cost [24, 25], BsPs represent promising candidates for antifibrotic drug development.

Despite the identification of various bioactive polysaccharides, their potential anti-hepatic fibrosis effects remained insufficiently explored, limiting their therapeutic applications. Prior investigation of *B. striata* cultivated in Zunyi revealed that crude polysaccharides extracted using ultrasound, enzymes, and thermal methods contained a Man Glu ratio of approximately 1:1, a composition distinct from previously reported compositions [26], suggesting the presence of novel BSP structures. Notably, the antifibrotic properties of this 1:1 glucomannan polysaccharide remained uncharacterized. Therefore, explicating its isolation, structural features, and impact on hepatic fibrosis would clarify its therapeutic utility. Based on these observations, we hypothesized that the predominant glucomannans from cultivated *B. striata* might exhibit antihepatic fibrosis activity. In this study, we isolated a novel glucomannan (BsPsIII1-1) with a molecular weight (Mw) of approximately 74.8 kDa from *B. striata*, which markedly attenuated TGF- β 1-induced fibrosis in LX2 cell and alleviated CCl₄ induced liver injury and fibrosis in mice. Mechanistically, BsPsIII1-1 mitigated hepatic fibrosis by suppressing the TGF- β 1/SMAD signaling pathway and modulating the gut microbiota. Specifically, it promoted the proliferation of beneficial bacteria taxa while reducing the harmful species, thereby synergistically enhancing anti-fibrotic effects through the gut-liver axis. These findings suggest that BsPsIII1-1 represents a promising candidate for antifibrotic therapy.

2. Materials and methods

2.1 Materials and reagents

Fresh rhizome of *B. striata* (Thunb. ex A. Murray) Rhb. f. was harvested in Zheng'an County, Zunyi, Guizhou, China (28.5592 °N, 107.4610 °E), and authenticated. The human HSC line LX-2 was procured from

Suzhou Beina Chuanglian Biotechnology Co., Ltd. Male C57BL/6J mice (weight: 18-20 g, 6-8 weeks) for this study were obtained from Sibeifu (Beijing) Biotechnology Co., Ltd. (License No: SCXK (Jing) 2024-0001).

DEAE-cellulose (DEAE-52) was procured from Shanghai Shifeng Biotechnology Co., Ltd., China. Sephadex G-150 and T-series dextrans (T-1, T-7, T-50, T-100, and T-200) were obtained from Pharmacia (Sweden). High-purity standard monosaccharides ($\geq 97\%$), including Man, galacturonic acid (GalA), Glc, galactose (Gal), Ara, and fucose (Fuc) were sourced from BioDuly, (Nanjing, China). 1-Phenyl-3-methyl-5-pyrazolone (PMP) and deuterium oxide (D_2O , $> 99.8\%$ purity) were purchased from J&K Chemical Ltd., (Beijing, China). Congo red ($> 98.0\%$ purity, HPLC) was acquired from Chengdu Kelong Chemical Factory, (Chengdu, China), while Picrosirius Red Staining Solution was obtained from Fuzhou Feijing Biotechnology Co., Ltd., (Fuzhou, China). The Modified Masson Tricolor Staining Kit, Enhanced DAB Chromogenic Kit ($20\times$), and assay kits for malondialdehyde (MDA), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and reduced glutathione (GSH) were acquired from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). Primary and secondary antibody dilution solutions for Western blotting (WB), RIPA lysis buffer (strong), SDS-PAGE protein sample loading buffer ($5\times$), and Tween 20 were obtained from Shanghai Beyotime Biotechnology Co., Ltd., (Shanghai, China). Rabbit monoclonal antibodies targeting α -SMA, transforming growth factor-beta1 ($TGF-\beta 1$), and SMAD family proteins (SMAD2, p-SMAD2, SMAD4, SMAD7) were sourced from Hangzhou Hua'an Biotechnology Co., Ltd., while the COL-1 antibody was procured from Abcam (Cambridge, UK). Ultrapure water was produced using a Milli-Q system (Millipore, Bedford, MA, USA). Silymarin, CCl_4 , and corn oil were sourced from Shanghai McLean Biochemical Technology Co., Ltd., (Shanghai, China). Methanol, 1-propanol, pyridine, hexane, propyl chloroformate were purchased from ANPEL. Sodium hydroxide and ethyl acetate were purchased from Sinopharm. HPLC-grade acetonitrile was provided by Fisher Scientific Worldwide Co., Ltd., (Shanghai, China). All other chemicals and solvents used were of analytical grade.

2.2 General experimental procedures

The aqueous solution was concentrated using an EYELA N-130 rotary evaporator (Eyela Instrument Co., Ltd., Shanghai, China). Polysaccharide samples were centrifuged at low speeds ($\leq 2,800 \times g$) using an HDL-40B centrifuge system from Changzhou Hongke Instrument Factory (China) and at high speeds using a Thermo Scientific ST8R centrifuge (USA). Freeze-drying of polysaccharide samples was performed using a Telstar LyoQuest freeze dryer from Azbil Telstar Technologies SLU (Spain). Absorbance measurements were conducted using a SpectraMax® i3x fluorescence microplate reader (Molecular Devices) in a 96-well plate. Dialysis was performed using a semipermeable membrane (MWCO 3,500 Da, Union Carbide Corporation, America). Microscopic morphological analysis was performed using an Olympus IX73 microscope (Tokyo, Japan).

2.3 Extraction, preliminary purification and separation of polysaccharide

The dried rhizome of *B. striata* was ground and subjected to triple extraction using 95% food-grade ethanol to remove small molecule impurities. The resulting residue was dried at $45^\circ C$, and subsequently

extracted with distilled water at a solid-to-liquid ratio of 1:30 (w/v) through ultrasonic-assisted extraction in an ice bath for 2 hours. The extract was filtered, and the residue was re-extracted twice. The combined filtrate was concentrated to 20%-30% of its original volume under reduced pressure. Polysaccharides were precipitated by adding four volumes of 95% ethanol, redissolved in water and centrifuged to collect the supernatant. Low-Mw compounds were removed via dialysis for 72 hours, and the crude polysaccharide was obtained by freeze-drying and stored at 4°C for future use.

The polysaccharide was dissolved in 50 mL of distilled water at a concentration of 500 mg/mL, and subjected to chromatography on a DEAE-52 column (270×60 mm). The elution was performed sequentially with water and 0.15 M NaCl at a flow rate of 1.0 mL/min. Eluates were collected in 10 mL tubes using an auto-fraction collector (Bio-Rad, USA) and analyzed at 490 nm employing the phenol-sulfuric acid colorimetric method [27]. The elution profile was generated by plotting absorbance against tube number, with peak fractions collected for further processing. These fractions were dialyzed for 72 hours to remove residual salt, and subsequently purified on a Sephadex G-150 column (600×35 mm) using deionized water at 0.3 mL/min (5 mL/tube). Fractions corresponding to elution peaks were collected and lyophilized for storage. Polysaccharide samples were imaged using a Huawei Enjoy 60X camera (Shenzhen, China). The extraction, purification, and separation procedures were summarized in Fig. 1.

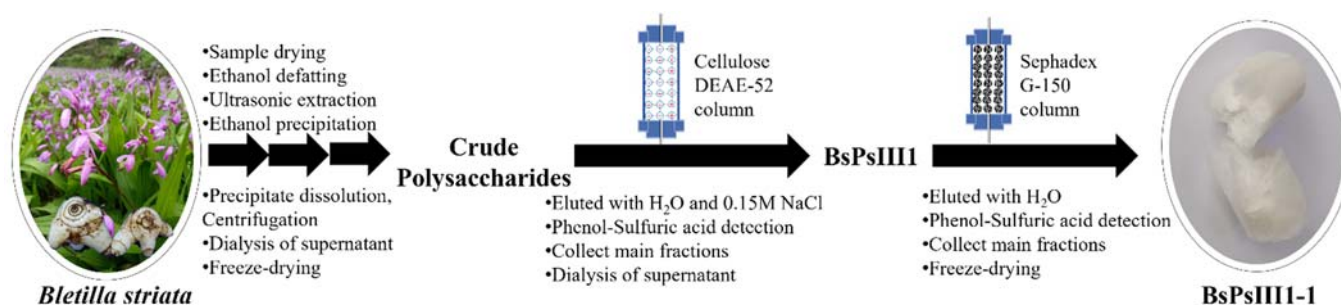


Fig.1. Crucial procedures related to the extraction, purification and separation of polysaccharide from *B. striata*

2.4 Ultra violet (UV) spectroscopic and relative Mw analysis

The UV absorption spectrum of the polysaccharide solution (1.0 mg/mL) was recorded using a Multiskan Go microplate reader (MGM) over the wavelength range of 200-800 nm.

The Mw of the polysaccharide was determined using a Daojing LC-20AT high-performance gel permeation chromatography (HPGPC) system (Shimadzu, Kyoto, Japan), which was equipped with a TSK-GEL GMPWxl column (7.8 mm × 30.0 cm) and an evaporative light scattering detector (ELSD; Alltech Associates, Deerfield, IL, USA). The methodology employed was consistent with our previous report [28]. Polysaccharide purity was assessed by analyzing the number, morphology, and relative distribution of major chromatographic peaks. A series of standard polysaccharides (T-series dextrans: T-1, T-7, T-50, T-100, and T-200, at 1.0 mg/mL) were selected for calibration. The HPGPC-ELSD analysis was performed under standardized conditions to construct a calibration curve by plotting the logarithm of Mw of the standard dextrans (lgMw) against the logarithm of the retention time (lgRT). Subsequently, the Mw of the

polysaccharide was determined based on its RT. Samples (0.5-1.0 mg/mL) were analyzed with an injection volume of 5.0 μ L.

2.5 Determination of the total carbohydrate, total protein and total uronic acid contents

Total carbohydrate content was quantified using the phenol-sulfuric acid method with Glc as the reference standard, in accordance with the protocol established by Dubois *et al.* [29] with slight modifications. Briefly, a mixture of 60 μ L phenol and 1.5 mL concentrated sulfuric acid was prepared, followed by the addition of 100 μ L of Glc (0.1-1.0 mg/mL) or the polysaccharide sample was added. This mixture was then incubated at 25°C for 60 minutes. The carbohydrate content was calculated from the absorbance values using the calibration curve: $Y = 1.1183X + 0.2525$, with an R^2 value of 0.9953, where Y represents absorbance and X indicates the total carbohydrate content.

Total protein content was quantified using the Bicinchoninic acid (BCA) assay following the protocol described by Walker [30]. Specifically, protein content was determined by employing the calibration curve: $Y = 0.9708X + 0.1946$, with an R^2 value of 0.9986, where Y represents the absorbance of the sample tested and X indicates the total protein content.

Uronic acid content was determined using the m-hydroxydiphenyl method outlined by Filisetti-Cozzi and Carpita [31]. The absorbance values were utilized to determine the uronic acid content based on the calibration curve: $Y = 3.542X + 0.0461$, with an R^2 value of 0.9918, where Y represents the absorbance of the sample and X denotes the total uronic acid content.

2.6 Monosaccharide composition analysis

The monosaccharide composition was analyzed using high-performance liquid chromatography (HPLC) with pre-column derivatization using PMP, following a modified protocol based on by Pan *et al.* [32]. Briefly, 2.0 mg of polysaccharide sample was hydrolyzed with 0.5 mL of 3 M trifluoroacetic acid (TFA) at 95°C for 6 hours in a nitrogen-purged, sealed 10-mL ampoule. After cooling, the sample was centrifuged at 12,000 $\times$ g. For derivatization, 200 μ L of the hydrolysate or standard monosaccharides (Man, Glc, GalA, Gal, Ara, and Fuc) was mixed with 300 μ L of 0.3 M NaOH and subsequently derivatized with 0.5 M PMP (300 μ L), with the resulting solution incubated at 70°C for 1 hours. The PMP derivatives were analyzed using an Agilent 1260 HPLC system (Agilent Technologies) equipped with a Supersil AQ-C18 column (250 $\times$ 4.6 mm i.d., 5 μ m, Elite Analytical Instruments Co., Ltd., Dalian, China). Elution was carried out with a mobile phase consisting of 17% acetonitrile and 83% phosphate-buffered saline (PBS, 0.05 mM, pH 7.2, v/v) at a flow rate of 1 mL/min and a column temperature of 30°C. Detection was carried out at 245 nm with an injection volume of 10 μ L.

2.7 Methylation analysis

Methylation analysis was performed according to established protocols with minor modifications [33-35]. Briefly, 2.0 mg of lyophilized polysaccharide was placed in a sealed vial and subjected to ultrasonication in 0.3 mL of anhydrous DMSO for 2-3 minutes under nitrogen, followed by a 30 minutes incubation at

room temperature. Subsequently, a suspension of NaOH in DMSO (0.3 mL) and methyl iodide (0.3 mL) was added. The reaction mixture underwent three cycles of ultrasonication (20 minutes each) and vortex mixing (5 minutes each). Upon completion of the reaction, 4 mL of deionized water was added to halt the methylation process. The methylated product was extracted with chloroform (1:1, v/v), followed by centrifugation at 4,000 $\times$ g. The lower chloroform layer was collected, and the extraction was repeated thrice. An equivalent volume of chloroform was added to the upper aqueous layer, and after shaking and centrifugation, this phase separation process was reiterated thrice. The combined chloroform phases were concentrated under reduced pressure, and the methylation procedure was performed three additional times to ensure complete derivatization.

For hydrolysis, the methylated polysaccharide was treated with 1 mL of 90% (v/v) formic acid at 90°C for 5 hours in a sealed tube. Following the evaporation of formic acid under reduced pressure, the residue was dissolved in 1 mL of 2 M TFA and hydrolyzed at 90°C for 6 hours in a sealed ampoule. The hydrolysate was then dissolved in methanol and evaporated to dryness under reduced pressure, with this step repeated three times to ensure complete removal of TFA. The sample was subsequently dissolved in 2 mL of 2 M ammonia solution, followed by the addition of 20 mg of NaBH₄ to initiate a reduction reaction at 37°C for 4 hours. Upon completion of the reaction, acetic acid was added dropwise until effervescence ceased, and the mixture was evaporated under reduced pressure to dryness. The residue was further evaporated with methanol (1.5 mL) containing a drop of acetic acid at temperatures below 40°C, repeating this process 4 to 5 times to remove borate residues. Finally, the product was treated with pure methanol to yield the dried reduced derivative.

For acetylation, 0.25 mL of pyridine and 0.75 mL of acetic anhydride were added to the reaction mixture, which was subsequently incubated at 37°C for 2-3 hours. The reaction was terminated by adding 2 mL of water and the resulting mixture underwent triple extraction with an equal volume of chloroform. The combined chloroform layers were washed three times with deionized water to eliminate impurities, followed by drying via rotary evaporation and further desiccation over silica gel. The dried sample was dissolved in 0.3 mL of chloroform to yield partially methylated alditol acetates (PMAA) for subsequent gas chromatography-mass spectrometry (GC-MS) analysis. A control sample without polysaccharides was processed in an identical manner for comparative analysis.

GC-MS analysis was performed using an ISQ-TRACE 1310 system (Thermo Fisher Scientific, Waltham, USA) equipped with electron ionization (EI) and a J&W DB-5ms gas chromatography column (30 m $\times$ 0.25 mm $\times$ 0.25 μ m, Agilent, USA). Injection volume was 1 μ L with a 15:1 split ratio, and helium was used as the carrier gas at a flow rate of 1.0 mL/min. The oven temperature program was as follows: initial hold at 100°C for 3.0 minutes, followed by a ramp at 10°C/min to 140°C (held for 2 minutes), then a second ramped at 3°C/min to 230°C, (held for 3 minutes). Detection was performed in full scan mode (m/z 50-400) using an EI source (70 eV). PMAA peaks were identified based on mass spectra, fragmentation patterns, and relative

retention times, with reference to the Complex Carbohydrate Research Center (CCRC) database (<https://www.ccrcc.uga.edu>).

2.8 Nuclear magnetic resonance spectroscopy analysis

For nuclear magnetic resonance (NMR) spectroscopy, a dried polysaccharide sample weighing approximately 25 mg was dissolved in 1.0 mL of D₂O. The sample underwent freeze-drying to replace H₂O with D₂O, and was subsequently reconstituted in 0.7 mL of D₂O at room temperature for NMR analysis. Spectra were acquired using either an Agilent-400 MHz spectrometer (Agilent, USA) or an AVANCE NEO 600 MHz spectrometer (Bruker, Billerica, MA, USA) for proton NMR (400 or 600 MHz), carbon NMR (125.48 MHz), proton-proton correlated spectroscopy (COSY, 600 MHz), proton-carbon heteronuclear single quantum coherence (HSQC, 600 MHz), proton-carbon heteronuclear multiple bond correlation (HMBC, 600 MHz), and proton-proton rotating frame nuclear Overhauser enhancement spectroscopy (ROESY, 600 MHz).

2.9 Congo-red and scanning electron microscope analysis

The Congo red test was performed following modifications to the protocol established by Zhang *et al.* [36]. Briefly, polysaccharide solutions at a concentration of 2.5 mg/mL were combined with 0.08 mM Congo red in NaOH solutions resulting in final NaOH concentrations ranging from 0.0 to 0.5 M. Following a 20-minute incubation period, the solutions were analyzed spectrophotometrically to determine the maximum absorption wavelength (λ_{max}).

For morphological characterization, polysaccharide samples were sputter-coated with gold under vacuum conditions. The morphology of polysaccharide samples was then analyzed using a Hitachi SU801 scanning electron microscope (SEM) (Hitachi High-Technologies, Japan). The SEM operated at an acceleration voltage of 3 kV, with images magnifications of 1,000 $\times$, 5,000 $\times$, and 10,000 $\times$.

2.10 Cell experiment

The human HSC line LX-2 was cultured in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C with 5% CO₂. For the anti-fibrotic study, cells were seeded at a density of 1.5×10^5 cells/well in 6-well plates, serum-starved and subsequently stimulated with 20 ng/mL TGF- β 1 for 48 hours to induce activation, with or without treatment with BsPsIII1-1. Untreated cells served as the control. A parallel cytotoxicity assay was performed by seeding cells at a density of 1×10^4 cells/well in a 96-well plate and treating them with increasing concentrations of BsPsIII1-1 (0.1–4 mg/mL) for 24 hours. Following the respective treatment periods, cell viability was assessed using the Sulforhodamine B (SRB) assay, which measures cellular content by staining fixed cells with SRB dye and quantifying absorbance at 530 nm.

2.11 Animal experiment

The experimental design is depicted in Fig.6A. After a one-week acclimatization period, fifty male C57BL/6J mice aged (6–8 weeks old, 18–20 g) were randomly divided into the normal, model, positive control (treated with 50 mg/kg silymarin via gavage daily), and BsPsIII1-1 (treated with 100 mg/kg or 400 mg/kg via gavage daily) groups (n=10). Except for the normal group, all mice were intraperitoneally injected with a 20%

CCl₄ corn oil solution (5 mL/kg, twice weekly for 6 weeks). The normal and model control groups were given an equivalent volume of saline via gavage daily. The body weight of each group was assessed and documented weekly at a consistent time. The animal experiment was approved by the Animal Research Ethics Committee of ** Medical University (Approval No. *21-2303-017) and followed institutional guidelines.

2.12 Hepatic index, AST, ALT, MDA and GSH measurement

At the study endpoint, body weight and liver weight were measured, and the hepatic index was calculated as (liver weight/body weight) × 100%. Blood samples were centrifuged at 3,500 ×g for 5 minutes to isolate serum. The levels of AST and ALT were quantified using commercial assay kits according to the manufacturer's protocols. Additionally, mouse liver tissue samples were homogenized in ice-cold buffer at a 1:10 (w/v) ratio and thoroughly vortexed. The homogenate was then centrifuged at 10,000 ×g for 10 minutes at 4°C. The resulting supernatant was preserved at 4°C for subsequent quantification of MDA and GSH levels using assay kits.

2.13 Histopathological examination

Liver tissue samples from the middle lobe were fixed in 4% neutral-buffered formalin for 24 hours, paraffin-embedded, and sectioned to a thickness of 4 µm. Following deparaffinization and rehydration through a graded ethanol series, the sections were stained with hematoxylin and eosin (H&E), Masson's trichrome, and Sirius red according to standard protocols as per the manufacturer's guidelines. The stained sections were then dehydrated, mounted with neutral gum, and examined under a light microscope.

2.14 Immunohistochemistry assay

Tissue sections underwent antigen retrieval in sodium citrate buffer, followed by endogenous peroxidase blocking with 3% H₂O₂ for 15 minutes. Subsequently, primary antibodies were applied for 70 minutes at room temperature, after which the sections were washed three times with PBS for 3 minutes each. Secondary antibody incubation was performed for 40 minutes at room temperature. After 3,3'-diaminobenzidine (DAB) chromogenic development for 5 minutes and hematoxylin counterstaining, the sections were differentiated, dehydrated, and mounted for microscopic examination.

2.15 Enzyme-linked immunosorbent assay (ELISA)

Serum TGF-β levels were quantified using a double antibody sandwich ELISA kit (ED-20862, LunChangShuo Biological, China) according to the manufacturer's protocol. Prior to loading onto a 96-well plate, serum samples were centrifuged, acidified, and neutralized alongside standards. Subsequent steps included the sequential addition of the detection antibody and enzyme conjugate. Following incubation and washing procedures, the TMB substrate was added for color development, and the reaction was terminated with stop solution. Absorbance was recorded at 450 nm using a microplate reader, and sample concentrations were determined via standard curve interpolation.

2.16 Western blotting analysis

Liver LX2 cells and animal tissue samples were homogenized in ice-cold RIPA buffer containing protease and phosphatase inhibitors. The samples were lysed on ice for 30 minutes, and subsequently centrifuged at 12,000 ×g for 15 minutes at 4°C. Protein concentrations were assessed using a BCA assay, and equal amounts of protein were loaded onto 10% SDS-polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes, which were blocked for 15 minutes, and incubated overnight at 4°C with primary antibodies. After washing with PBS containing 0.1% Tween-20 (PBST), the membranes were incubated with secondary antibodies for 2 hours at room temperature. Protein bands were visualized using a dye-free rapid protein blotting system, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) serving as the loading control. Quantitative analysis was performed using ImageJ software.

2.17 Gut microbiota 16S rRNA sequencing assay

One week prior to mouse dissection, fecal samples were collected and stored at -80°C. 16S rRNA sequencing targeting the V3-V4 region was conducted by Sanshu Biotechnology Co., Ltd. (Jiangsu, China). The V3-V4 hypervariable region of the bacterial 16S rRNA gene was amplified using universal primers (forward: 5'-ACTCCTACGGGAGGCAGCAG-3' and reverse: 5'-GGACTACHVGGGTWTCTAAT-3'). Genomic DNA was extracted from fecal specimens according to the instructions of the E.Z.N.A.® soil DNA kit (Omega Bio-tek, Norcross, GA, U.S.) and subsequently underwent by PCR amplification. Amplicons were verified by 2% agarose gel electrophoresis, purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA), and quantified with a Quantus™ Fluorometer (Promega, Madison, WI, USA). Sequencing libraries were prepared using the NEXTflex™ Rapid DNA-Seq Kit (Bioo Scientific, USA), and sequenced on the Illumina NovaSeq PE250 platform (Illumina, San Diego, CA, USA). Operational taxonomic units (OTUs) were clustered using UPARSE software (<http://drive5.com/uparse/>, version 7.1) [37], and taxonomic classification was performed with the RDP Classifier (<http://rdp.cme.msu.edu/>, version 2.2) [38].

2.18 Determination of short-chain fatty acids

Short-chain fatty acids (SCFAs) were analyzed using GC-MS. Calibration standards were prepared at concentrations of 1, 10, 20, 50, and 100 µg/mL for acetic, butyric, isobutyric, valeric, isovaleric, hexanoic, and 4-methylvaleric acids, and 1, 45, 100, 200, and 300 µg/mL for propionic acid. For sample preparation, approximately 50 mg of fecal sample was homogenized with a mixture of 50 µL of 30% phosphoric acid and 300 µL of acetone for 3 minutes. Subsequently, the homogenate was centrifuged at 12,000 rpm for 10 minutes, and the resulting supernatant was diluted and analyzed using an Agilent 7820A GC coupled with an Agilent 5977B mass spectrometer (Agilent Technologies, USA). Chromatographic separation was achieved using a DB-FFAP capillary column (30 m × 0.25 mm × 0.25 µm), with a sample injection volume of 1 µL and a split ratio of 10:1. High purity helium served as the carrier gas at a constant flow rate of 1.0 mL/min. The oven temperature program initiated at 70°C for 5 minutes, followed by a ramp of 6°C/min to reach 100°C. Mass spectrometry detection employed an EI source operating at 70 eV, with the ion source and quadrupole

temperatures maintained at 260°C and 150°C, respectively. Data were acquired in selected ion monitoring (SIM) mode (m/z 30-550) and quantified using external calibration curves in Quant-My-Way software.

2.19 Statistical analysis

Data are presented as mean $\pm$ standard deviation (SD) from a least minimum of three independent experiments. For *in vivo* animal studies, data were obtained from a representative subset of randomly selected samples from the complete cohort to ensure objectivity and representativeness. Sample selection adhered to randomized principles to eliminate selection bias. Statistical analyses were performed using SPSS 29.0 (IBM Corp., Armonk, NY, USA). Comparisons among multiple groups were conducted using one-way analysis of variance (ANOVA), while pairwise comparisons employed a T-test. Statistical significance levels were set at $P < 0.05$ (significant), $P < 0.01$ (highly significant), and $P < 0.001$ (extremely significant). Graphs were generated using Microsoft Office Excel 2010 (Microsoft Corp., USA) or Origin Pro 8 (OriginLab, USA), and quantitative image analysis was performed with ImageJ (National Institutes of Health, USA).

3. Results

3.1 Extraction, purification and isolation of polysaccharide

In this study, we obtained polysaccharides (designated BsPsIII) extracted from 3-year cultivated *B. striata* were obtained through low-temperature ultrasonic-assisted extraction, ethanol precipitation, and freeze-drying, yielding approximately 38% (Fig.1). To achieve homogeneity, we subjected the crude BsPsIII to sequential purification using DEAE-52 and Sephadex G-150. The DEAE-52 chromatogram (Fig.2A) revealed a predominant peak accompanied by several minor peaks, with the major fraction collected from tubes 16-29, designated as BsPsIII1. Following desalting via dialysis, BsPsIII1 was further purified on a Sephadex G-150 column, yielding a distinct fraction (BsPsIII1-1) from tubes 21-42 (Fig.2B). We confirmed the homogeneity of BsPsIII1-1 was confirmed by the absence of UV absorption at 260 nm and 280 nm as well as the presence of a single symmetrical peak in HPGPC chromatogram coupled with ELSD (Figs.2C and 2D), indicating a uniform Mw distribution^[39].

3.2 Homogeneity and Mw of BsPsIII1-1

The polysaccharide fraction (BsPsIII1-1) was dissolved in ultrapure water and analyzed using HPGPC-ELSD. We generated a calibration curve using T-series dextran standards, plotting the natural logarithm of the Mw against RT^[40]. We determined the Mw was determined from the RT value using the established calibration curve: $\ln(\text{Mw}) = -18.815\ln(\text{RT}) + 62.888$, with an R^2 value of 0.9745. As shown in Fig.2D, a single symmetrical peak at 15.613 min on the HPGPC-ELSD chromatogram corresponded to a calculated Mw of approximately 74.8 kDa.

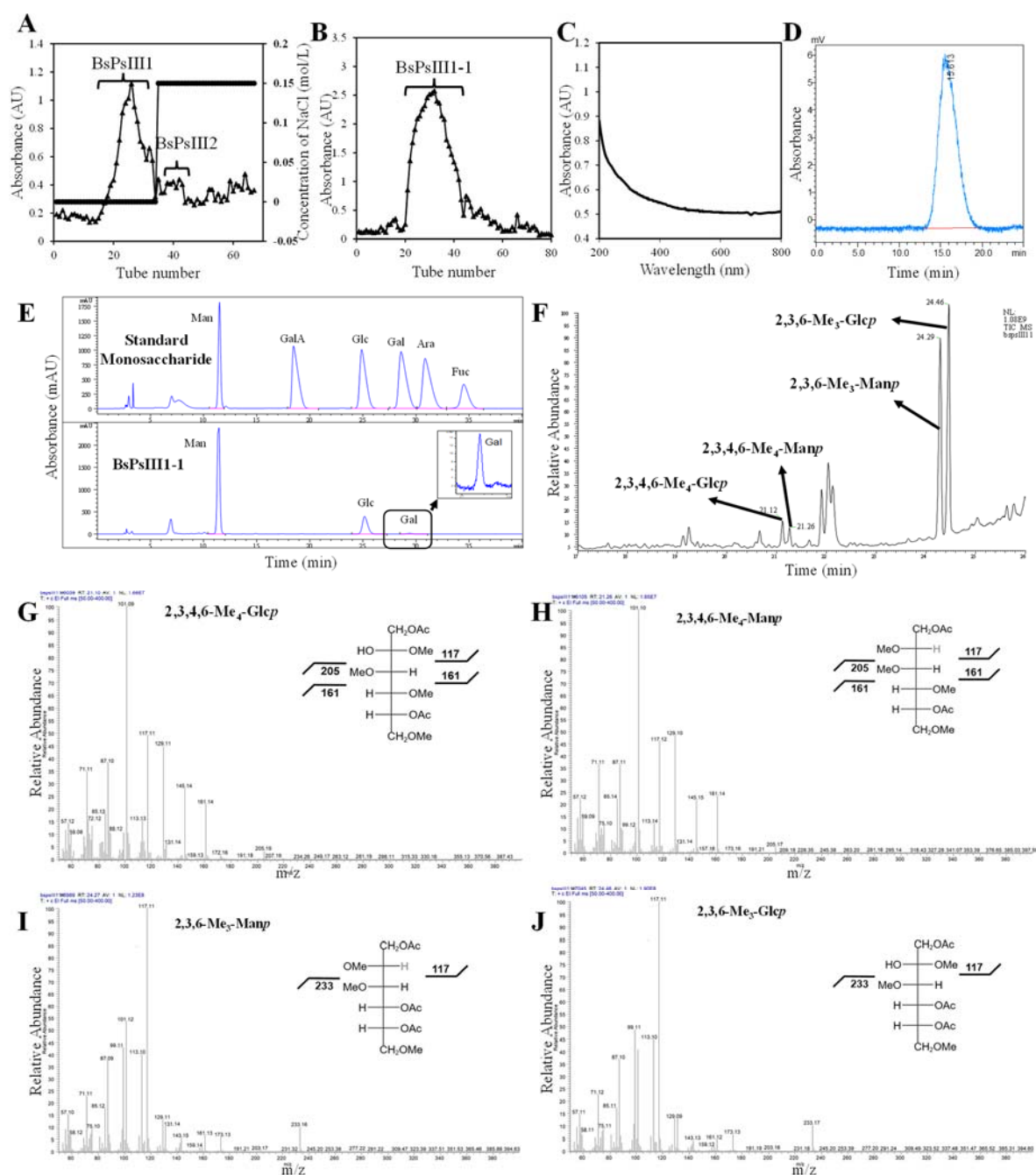


Fig. 2. Elution chromatograms of the crude polysaccharides from *B. striata* (BsPs) on a DEAE 52-cellulose column (A), on a Sepharose G-150 gel filtration column (B), the UV-vis spectrum (C), HPGPC-ELSD profile (D), and PMP precolumn derivatization HPLC chromatogram of the hydrolyzed monosaccharides (E), the PMAAs' GC-MS chromatograms of BsPsIII1-1 (F), MS fragments of PMAAs of BsPsIII1-1 and deduced residues (G-J) (G, 1,5-Di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol (2,3,4,6-Me₄-Glcp), H, 1,5-Di-O-acetyl-2,3,4,6-tetra-O-methyl-D-mannitol (2,3,4,6-Me₄-Manp), I, 1,4,5-Tri-O-acetyl-2,3,6-tri-O-methyl-D-mannitol (2,3,6-Me₃-Manp), and J, 1,4,5-Tri-O-acetyl-2,3,6-tri-O-methyl-D-mannitol (2,3,6-Me₃-Glcp).

Note: Man, mannose; GalA, galacturonic acid; Glc, glucose; Gal, galactose; Ara, arabinose; Fuc, fucose.

3.3 Chemical composition of BsPsIII1-1

As summarized in Table 1, compositional analysis of BsPsIII1-1 revealed $93.80\% \pm 4.65\%$ total carbohydrate content, undetectable protein levels ($-0.37\% \pm 4.63\%$), and $3.48\% \pm 1.86\%$ uronic acid. The uronic acid content, determined by the m-hydroxydiphenyl method, was below 5% with a relatively large standard deviation ($\pm 1.86\%$), suggesting that this trace amount likely falls near the detection limit of the assay

and may reflect slight interference from neutral sugars or nonspecific chromophores rather than a true acidic sugar moiety. Collectively, these results confirm that BsPsIII1-1 is a neutral polysaccharide lacking protein conjugation.

3.4 Monosaccharide composition of BsPsIII1-1

Monosaccharide composition plays a pivotal role in the structural analysis of polysaccharides and their associated bioactive investigations [41]. BsPsIII1-1 was found to consist of Man, Glc and Gal at a molar ratio of 100.00:88.17:2.32 (Fig.2E and Table 1). Compositional analysis indicated that BsPsIII1-1 contained Man and Glc in approximately a 1:1 ratio, collectively accounting for more than 98% of the total content.

Table 1 Chemical characteristics, and monosaccharide composition of BsPsIII1-1

Polysaccharide	Total sugar (%)	Protein (%)	Uronic acid (%)	Monosaccharide composition ^a		
				Man	Glc	Gal
BsPsIII1-1	93.80±4.65	-0.37±4.63	3.48±1.86	100.00	88.17	2.32

Note: ^a, Percent molar ratio of the sugar residues in the obtained polysaccharide (% of molar ratio); Man, mannose; Glc, glucose; Gal, galactose.

3.5 Methylation-based structural analysis of BsPsIII1-1

In this study, we identified four partially methylated alditol acetates were identified through electron ionization mass spectrometry (EI-MS) fragmentation patterns matched against the CCRC database (Table 2, Figs. 2F-J): 2,3,4,6-Me₄-Glc_p (5.36%), 2,3,4,6-Me₄-Man_p (4.26%), 2,3,6-Me₃-Man_p (43.77%), and 2,3,6-Me₃-Glc_p (46.60%). The predominant derivatives were 2,3,6-Me₃-Man_p (43.77%) and 2,3,6-Me₃-Glc_p (46.60%), indicating that BsPsIII1-1 primarily consisted of →4)-Man_p-(1→ and →4)-Glc_p-(1→ residues in a nearly 1:1 molar ratio, which was consistent with the previous monosaccharide.

Additionally, we calculated the degree of branching (DB) of BsPsIII1-1 was calculated using the formula $DB = (N_t + N_b)/(N_t + N_b + N_l)$, where N_t , N_b , and N_l denoted the total number of terminal residues, branched residues, and linear residues, respectively. The calculated DB value of 9.62% indicated a relatively low degree of branching, consistent with a predominantly linear structural configuration.

Table 2. GC-MS analysis of methylated BsPsIII1-1

Retention time (min)	Linkages patterns ^a	Mass fragments (m/z)	Methylated sugars ^b	Relative amount (%)
21.12	T-d-Glc _p -(1→	71, 87, 101, 117, 129, 161, 191, 205	2,3,4,6-Me ₄ -Glc _p	5.36
21.26	T-d-Man _p -(1→	71, 87, 101, 117, 129, 161, 191, 205	2,3,4,6-Me ₄ -Man _p	4.26
24.29	→4)-d-Man _p (1→	71, 87, 99, 101, 113, 117, 129, 161, 173, 233	2,3,6-Me ₃ -Man _p	43.77
24.46	→4)-d-Glc _p (1→	71, 87, 99, 101, 113, 117, 129, 161, 173, 233	2,3,6-Me ₃ -Glc _p	46.60

Note: ^a, Based on derived *O*-methylalditol Actates.

^b, Analyzed by GC-MS, after per-*O*-methylation, total acid hydrolysis, reduction, and Acetylation. 2,3,4,6-Me₄-Man_p= 1,5-di-*O*-Acetyl-2,3,4,6-tetra-*O*-methyl-mannose, etc.

3.6 NMR-Based structural analysis of BsPsIII1-1

The structural features of BsPsIII1-1 were further elucidated using 1D and 2D NMR spectroscopy (Fig.3). The ¹H NMR spectrum revealed chemical shifts in the anomeric region ranging from δ 4.3-5.5 ppm,

with α -glycosidic bond proton signals at δ 5.0–5.5 ppm and β -glycosidic bond signals at δ 4.3–4.85 ppm [42]. Fig.3A depicted more than 10 distinct anomeric signals within the δ 4.3–5.5 ppm range, indicative of multiple different monosaccharide residues in BsPsIII1-1. In the ^{13}C NMR spectrum, anomeric carbon shifts were predominantly observed between δ 90–110 ppm [43]. Although we detected multiple anomeric carbon signals were detected (Fig.3B), signal overlap or undetected peaks might have limited resolution. We attributed carbon shifts at δ 57–87 ppm to C2–C6 carbons [42]. Specifically, signals at δ 65–70 ppm corresponded to unsubstituted C6 of sugar residues, indicating unsubstituted C6 positions [44], consistent with methylation analysis. We identified acetyl methyl protons (δ 1.98–2.20 ppm) and their corresponding carbons (δ 20.00–22.30 ppm). Analysis of the ^1H - ^{13}C HSQC spectrum (Fig.3D) revealed cross peaks between δ 1.88–2.20/20.00–22.30 ppm. Acetyl carbonyl signals were observed at δ 172.82–173.83 ppm in the ^{13}C NMR spectrum, supported by HMBC cross peaks between δ 1.98–2.20/174.10–174.85 ppm (Fig.3E) further validating the acetylation of BsPsIII1-1.

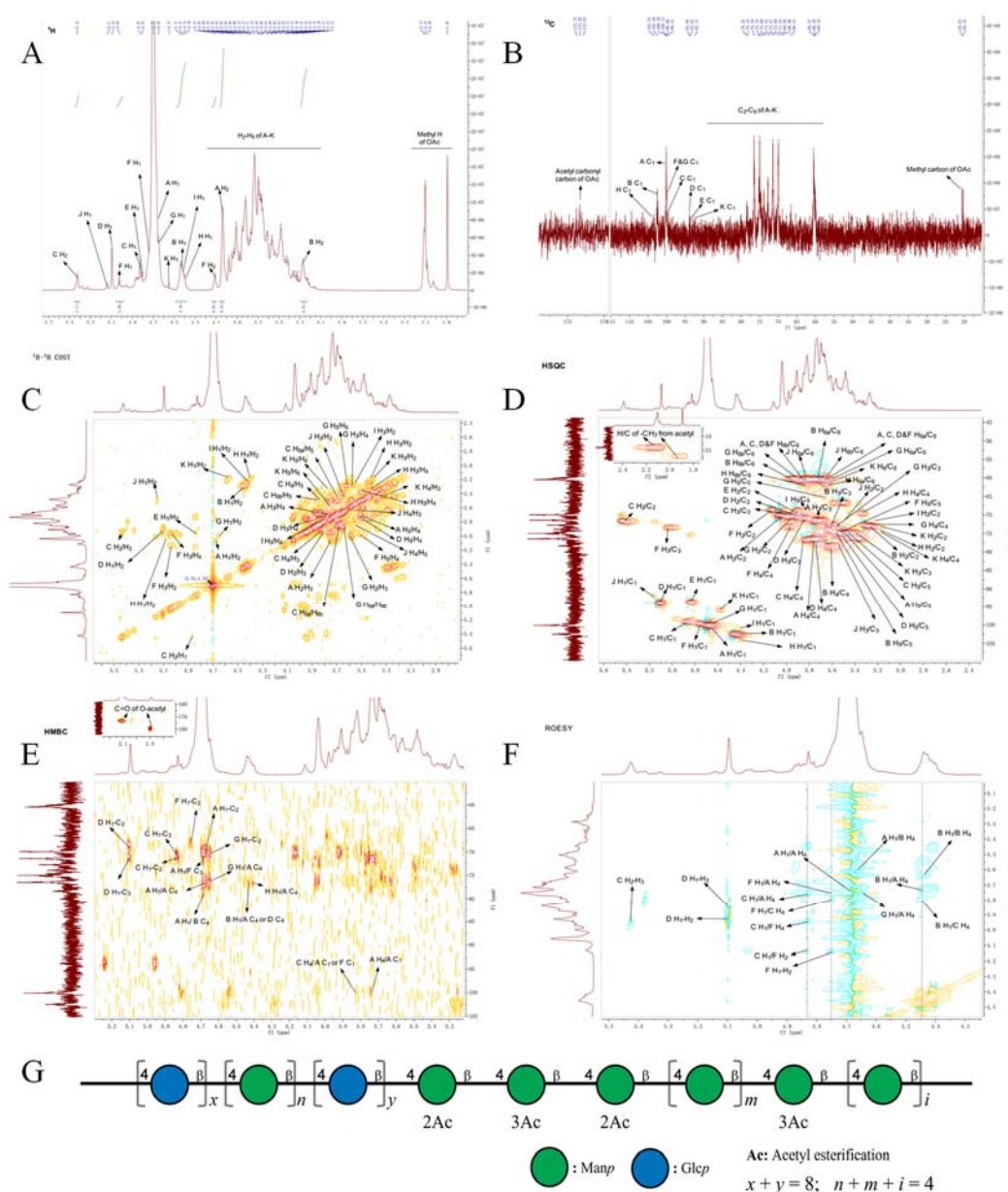


Fig.3. The NMR spectra (A, ^1H ; B, ^{13}C ; C, ^1H - ^1H COSY; D, ^1H - ^{13}C HSQC; E, ^1H - ^{13}C HMBC, F, ^1H - ^1H ROESY) and possible repeating unit structure (F) of BsPsIII1-1.

Methylation analysis identified 4 sugar residues, with the predominant residues identified as $\rightarrow 4$)- β -D-Manp-(1 $\rightarrow$ and $\rightarrow 4$)-D-Glcp-(1 $\rightarrow$. In the HSQC spectrum (Fig.3D), we observed a notable anomeric proton/carbon signal at δ 4.68/100.28 ppm (residue **A**). This signal, in accordance with previous studies [22, 45, 46], corresponds to the anomeric signal of $\rightarrow 4$)- β -D-Manp-(1 $\rightarrow$. Assignments of signals from H2 to H5 and C2 to C5 of residue **A** were determined to be δ 4.04 ppm, δ 3.74 ppm, δ 3.73 ppm, and δ 3.49 ppm, respectively, based on COSY analysis. Additionally, H6a and H6b were assigned chemical shifts of δ 3.68 ppm and δ 3.76 ppm, respectively, through COSY analysis and HSQC correlations (Figs.3C and 3D). We identified the chemical shifts of C2 to C6 in the sugar ring were identified as δ 70.01 ppm, δ 71.64 ppm, δ 76.52 ppm, δ 75.11 ppm, and δ 60.48 ppm, respectively, using the HSQC spectrum. Analysis of the HMBC spectrum revealed a heteronuclear long-range proton/carbon correlation at δ 4.68/70.01 ppm, which indicated a linkage between the H1 of residue **A** (δ 4.68 ppm) and the C2 of residue **A** (δ 70.01 ppm), consistent with the anticipated chemical environment for a β -linked Man residue. Comparative analysis with literature data [47] showed that the free β -D-Manp displayed C1 and C4 chemical shifts at δ 94.5 ppm and δ 67.7 ppm, respectively. By contrast, residue **A** exhibited significant downfield shifts at C1 and C4, with chemical shifts at δ 100.28 ppm and δ 76.52 ppm, respectively, which confirmed residue **A** as $\rightarrow 4$)- β -D-Manp-(1 $\rightarrow$.

The anomeric proton/carbon signal appeared prominently at δ 4.44/102.51 ppm (residue **B**) in the HSQC spectrum (Fig.3D), corresponding to the $\rightarrow 4$)- β -D-Glcp-(1 $\rightarrow$ anomeric signal as reported in previous studies [22, 45]. Assignments of H2 to H5 chemical shifts in residue **B** were determined as follows: δ 3.29 ppm, δ 3.61 ppm, δ 3.62 ppm, and δ 3.56 ppm. H6a and H6b were attributed to δ 3.74 ppm and δ 3.95 ppm, respectively, based on COSY and HSQC spectra (Fig.3C and 3D). The chemical shifts of C2 to C6 in the sugar ring of residue **B** were identified as δ 72.78 ppm, δ 74.04 ppm, δ 78.33 ppm, δ 75.02 ppm, and δ 60.40 ppm, respectively, using the HSQC spectrum. Comparison with data from the literature [47] revealed that although free β -D-Glcp displayed C1 and C4 chemical shifts at δ 96.7 ppm and δ 70.6 ppm, respectively, residue **B** exhibited significant downfield shifts at C1 (δ 102.51 ppm) and C4 (δ 78.33 ppm), indicating substitutions at these positions and confirming the identity of residue **B** as $\rightarrow 4$)- β -D-Glcp-(1 $\rightarrow$.

The ^1H NMR spectrum exhibited a prominent absorption peak at δ 5.42 ppm (residue **C**) and a distinct HSQC signal at δ 5.42/71.38 ppm. Additionally, we observed an absorption peak between δ 5.04 ppm (residue **F**) and a corresponding HSQC signal at δ 5.04/73.60 ppm. Notably, these signals were identified as nonanomeric: typical anomeric carbon signals appear above δ 90 ppm in ^{13}C NMR spectra [43]. Comprehensive analysis of monosaccharide composition, methylation data, and NMR spectroscopy indicated that BsPsIII1-1 contained at least 2 types of acetyl groups, which indicated acetylation substitution at residues **C** and **F**. Comparative analysis with prior studies [22, 45] revealed that C2 acetylation of $\rightarrow 4$)- β -D-Manp-(1 $\rightarrow$ led to a substantial downfield shift in H2 around δ 5.40 ppm. Consequently, the signal at δ 5.42/71.38 ppm in residue **C** was identified as $\rightarrow 4$)- β -D-2Ac-Manp-(1 $\rightarrow$. Validation of the chemical shifts of H1/C1 and H3/C3 in residue **C** (δ 4.85/99.51 ppm and δ 3.95/70.23 ppm, respectively) was achieved through COSY and HSQC spectra. Correlations at δ 4.85/71.38 ppm and δ 4.85/70.23 ppm in the HMBC spectrum as well as a

correlation at δ 5.42/3.95 ppm in the ROESY spectrum further supported the identification of residue **C** as $\rightarrow 4$)- β -D-2Ac-Manp-(1 $\rightarrow$. Similarly, C3 acetylation of $\rightarrow 4$)- β -D-Manp-(1 $\rightarrow$ resulted in a significant downfield shift in H3 to approximately δ 5.00 ppm. Analysis of COSY and HSQC spectra, along with the signal at δ 5.03 ppm in residue **F**, confirmed the chemical shifts of H2/C2 (δ 4.16/68.69 ppm) and H4/C4 (δ 3.96/73.89 ppm). HMBC correlation at δ 4.75/68.69 ppm and ROESY correlation at δ 4.75/4.16 ppm, supported the identification of residue **F** as $\rightarrow 4$)- β -D-3Ac-Manp-(1 $\rightarrow$ correlating with previous literature [22, 45, 48]. Further signal assignments were provided in Table 3.

Table 3 Chemical shift (δ) assignments of ^1H and ^{13}C NMR spectra of BsPsIII1-1 on the basis of ^1H - ^1H COSY, ^1H - ^{13}C HSQC, ^1H - ^{13}C HMBC and ^1H - ^1H ROESY spectra.

Glycosyl residues		H1	H2	H3	H4	H5	H6a, H6b
		C1	C2	C3	C4	C5	C6
A	$\rightarrow$ 4)- β -D-Manp-(1 $\rightarrow$	4.68	4.04	3.74	3.73	3.49	3.68, 3.76
		100.28	70.01	71.64	76.52	75.11	60.48
B	$\rightarrow$ 4)- β -D-Glcp-(1 $\rightarrow$	4.44	3.29	3.61	3.62	3.56	3.74, 3.95
		102.51	72.78	74.04	78.33	75.02	60.4
C	$\rightarrow$ 4)- β -D-2Ac-Manp-(1 $\rightarrow$	4.85	5.42	3.95	3.82	3.48	3.69,3.85
		99.51	71.38	70.23	77.22	75.16	60.35
D	$\rightarrow$ 4)- α -D-Manp	5.10	3.92	3.83	3.72	3.51	3.68, 3.85
		93.89	69.01	71.49	76.44	76.37	60.33
E	$\rightarrow$ 4)- β -D-Manp	4.81	3.96	-	-	-	-
		93.62	69.00	-	-	-	-
F	$\rightarrow$ 4)- β -D-3Ac-Manp-(1 $\rightarrow$	4.75	4.16	5.03	3.96	3.52	3.68, 3.85
		100.11	68.69	73.60	73.89	75.20	60.33
G	T- β -D-Manp	4.66	3.98	3.58	3.28	3.63	3.64,3.89
		99.90	71.48	72.92	72.43	65.25	60.01
H	T- β -D-Glcp	4.40	3.20	3.43	3.30	3.52	3.63,3.83
		104.12	74.56	76.46	69.47	75.05	61.43
I	T- β -D-Galp	4.39	3.21	3.56	3.78	3.85	3.56,3.73
		103.51	71.66	71.43	74.89	69.05	61.66
J	$\rightarrow$ 4)- α -D-Glcp	5.15	3.47	3.64	3.55	3.87	3.84,3.70
		92.31	71.92	72.15	78.1	69.40	59.86
K	$\rightarrow$ 4)- β -D-Glcp	4.57	3.17	3.41	3.32	3.75	3.58
		95.83	73.33	74.9	74.78	74.17	60.09
O-Acetyl group		172.82-173.83 (C=O)/ 20.00-22.30 (CH ₃)/ 1.98-2.20 (CH ₃)					

The HSQC spectrum (Fig.3D) revealed two distinct anomeric proton/carbon signals at δ 5.10/93.89 ppm (residue **D**) and δ 4.81/93.62 ppm (residue **E**), which corresponded to mannose reducing end groups. Referencing the literature [22] showed that these residues were identified as $\rightarrow 4$)- α -D-Manp and $\rightarrow 4$)- β -D-Manp, respectively. HMBC correlations at δ 5.10/69.01 ppm and δ 5.10/71.49 ppm confirmed the linkage between H1 of residue **D** (δ 5.10 ppm) and C2 and C3 of residue **D** (δ 69.01 ppm and δ 71.49 ppm). The ROESY spectrum further supported residue **D** as $\rightarrow 4$)- α -D-Manp by validating correlations at δ 5.10/3.92 ppm and δ 5.10/3.83 ppm. Additionally, we detected weaker anomeric proton/carbon signals at δ 5.15/92.36 ppm (residue **J**) and δ 4.57/95.38 ppm (residue **K**), consistent with glucose reducing end groups. Comparison with the literature [48] revealed that these residues correspond to $\rightarrow 4$)- α -D-Glcp and $\rightarrow 4$)- β -D-Glcp, respectively, indicating the presence of 4 distinct types of reducing end carbon residues at the termini of the BsPsIII1-1 sugar chain.

The HSQC spectrum revealed two additional anomeric region chemical shift signals at δ 4.66/99.90 ppm (residue **G**) and δ 4.44/104.12 ppm (residue **H**). Corresponding H1/H2 correlations were identified in the COSY spectrum at δ 4.66/3.98 ppm and δ 4.44/3.20 ppm, respectively. Methylation analysis demonstrated the presence of 2 terminal nonreducing sugar residues, T-D-Manp-(1 $\rightarrow$ and T-D-Glcp-(1 $\rightarrow$, which were subsequently confirmed as residues **G** and **H**, respectively, by comparing them with previous studies [22, 45]. Monosaccharide composition analysis revealed a minor Gal component in BsPsIII1-1, supported by an anomeric region signal at δ 4.39/103.51 ppm (residue **I**) in the HSQC spectrum and a corresponding cross peak at δ 4.39/3.21 ppm in the COSY spectrum. Comparative analysis of prior research [49] established residue **I** as T-D-Glcp-(1 $\rightarrow$). We determined the remaining signal assignments through comprehensive analysis of chemical shifts in COSY, HSQC, and HMBC spectra, cross-referenced with established literature [22, 45, 49], as summarized in Table 3.

The inter-residue linkage sites and sequences were elucidated through comprehensive analysis of HMBC and ROESY spectra (Figs.3E and 3F), which facilitated the identification of interresidual connections. C-H connectivities between sugar residues were identified and tabulated in Table 4. In the HMBC spectrum, we observed 3 cross-peaks: H1 of residue **A** (δ 4.68 ppm) correlated with C4 of residues **F** (δ 73.89 ppm), **A** (δ 76.52 ppm), and **B** (δ 100.28 ppm), and H4 of residue **A** (δ 3.73 ppm) correlated with C1 of residue **A** (δ 100.28 ppm), indicating 1,4-glycosidic bonds between residue **A** and residues **F**, **A**, and **B**. The ROESY spectrum further corroborated these findings, revealing correlations between H1 of residue **A** and H4 of residues **F**, **A**, and **B**, confirming the glycosidic sequence as $\rightarrow$ 4)- β -D-Manp-(1 $\rightarrow$ 4)- β -D-2Ac-Manp-(1 $\rightarrow$, $\rightarrow$ 4)- β -D-Manp-(1 $\rightarrow$ 4)- β -D-Manp-(1 $\rightarrow$, and $\rightarrow$ 4)- β -D-Manp-(1 $\rightarrow$ 4)- β -D-Glcp-(1 $\rightarrow$. For residue **B**, the HMBC spectrum displayed correlations between H1 of residue **B** (δ 4.44 ppm) and C4 of residues **A** or **D** (δ 76.50 ppm). In the ROESY spectrum, H1 of residue **B** (δ 4.44 ppm) correlated with H4 of residues **B** (δ 3.62 ppm), **A** (δ 3.73 ppm), and **C** (δ 3.82 ppm), with strong signals for **B** and **A** but no correlation with the H4 of **D**, indicating 1,4-glycosidic bonds between **B** and residues **B**, **A**, and **C** and suggesting the glycosidic sequence to be $\rightarrow$ 4)- β -D-Glcp-(1 $\rightarrow$ 4)- β -D-Glcp-(1 $\rightarrow$, $\rightarrow$ 4)- β -D-Glcp-(1 $\rightarrow$ 4)- β -D-Manp-(1 $\rightarrow$, and $\rightarrow$ 4)- β -D-Glcp-(1 $\rightarrow$ 4)- β -D-2Ac-Manp-(1 $\rightarrow$. A weak cross-peak was observed between H4 of residue **C** (δ 3.83 ppm) and C1 of residues **A** and **F** (δ 100.07 ppm), suggesting a possible 1,4-glycosidic bond between residue **C** and the C1 position of **A** or **F**. Strong correlations in the ROESY spectrum between H1 of residue **C** and H4 of residues **A** and **F** confirmed connections of residue **C** with **A** and **F** through 1,4-glycosidic bonds, indicating the glycosidic sequence as $\rightarrow$ 4)- β -D-2Ac-Manp-(1 $\rightarrow$ 4)- β -D-Manp-(1 $\rightarrow$ and $\rightarrow$ 4)- β -D-2Ac-Manp-(1 $\rightarrow$ 4)- β -D-2Ac-Manp-(1 $\rightarrow$.

Table 4. HMBC (long-range $^1\text{H}/^{13}\text{C}$) and ROESY (long-range $^1\text{H}/^1\text{H}$) connectivities of main glycosidic sequences in BsPsIII1-1

HMBC						
Sugar residue	Position	Paired position	$^1\text{H}/^{13}\text{C}$ connectivity (δ , ppm)	Paired sugar residue	Glycosidic sequence	Sugar residue

A	$\rightarrow 4$)- β -D-Manp-(1 $\rightarrow$	H1	4.68/73.89	C4	F	$\rightarrow 4$)- β -D-Manp-(1 $\rightarrow 4$)- β -D-2Ac-Manp-(1 $\rightarrow$
		H1	4.68/76.52	C4	A	$\rightarrow 4$)- β -D-Manp-(1 $\rightarrow 4$)- β -D-Manp-(1 $\rightarrow$
		H1	4.68/78.33	C4	B	$\rightarrow 4$)- β -D-Manp-(1 $\rightarrow 4$)- β -D-Glcp-(1 $\rightarrow$
		H4	3.73/100.28	C1	A	$\rightarrow 4$)- β -D-Manp-(1 $\rightarrow 4$)- β -D-Manp-(1 $\rightarrow$ $\rightarrow 4$)- β -D-Glcp-(1 $\rightarrow 4$)- β -D-Manp-(1 $\rightarrow$ or $\rightarrow 4$)- β -D-Glcp-(1 $\rightarrow 4$)- β -D-Manp
B	$\rightarrow 4$)- β -D-Glcp-(1 $\rightarrow$	H1	4.44/76.50	C4	A or D	$\rightarrow 4$)- β -D-Manp-(1 $\rightarrow 4$)- β -D-2Ac-Manp-(1 $\rightarrow$ or $\rightarrow 4$)- β -D-3Ac-Manp-(1 $\rightarrow 4$)- β -D-2Ac-Manp-(1 $\rightarrow$
C	$\rightarrow 4$)- β -D-2Ac-Manp -(1 $\rightarrow$	H4	3.83/100.07	C1	A or F	$\rightarrow 4$)- β -D-Manp-(1 $\rightarrow 4$)- β -D-2Ac-Manp-(1 $\rightarrow$ or $\rightarrow 4$)- β -D-3Ac-Manp-(1 $\rightarrow 4$)- β -D-2Ac-Manp-(1 $\rightarrow$

ROESY

Sugar residue	Position	Paired position	¹ H/ ¹ H connectivity (δ , ppm)	Paired sugar residue	Glycosidic sequence	Sugar residue
A	$\rightarrow 4$)- β -D-Manp-(1 $\rightarrow$	H1	4.68/3.62	H4	B	$\rightarrow 4$)- β -D-Manp-(1 $\rightarrow 4$)- β -D-Glcp-(1 $\rightarrow$
		H1	4.68/3.73	H4	A	$\rightarrow 4$)- β -D-Manp-(1 $\rightarrow 4$)- β -D-Manp-(1 $\rightarrow$
		H1	4.68/3.96	H4	F	$\rightarrow 4$)- β -D-Manp-(1 $\rightarrow 4$)- β -D-2Ac-Manp-(1 $\rightarrow$
B	$\rightarrow 4$)- β -D-Glcp-(1 $\rightarrow$	H1	4.44/3.62	H4	B	$\rightarrow 4$)- β -D-Glcp-(1 $\rightarrow 4$)- β -D-Glcp-(1 $\rightarrow$
		H1	4.44/3.73	H4	A	$\rightarrow 4$)- β -D-Glcp-(1 $\rightarrow 4$)- β -D-Manp-(1 $\rightarrow$
		H1	4.44/3.82	H4	C	$\rightarrow 4$)- β -D-Glcp-(1 $\rightarrow 4$)- β -D-2Ac-Manp-(1 $\rightarrow$
C	$\rightarrow 4$)- β -D-2Ac-Manp -(1 $\rightarrow$	H1	4.85/3.73	H4	A	$\rightarrow 4$)- β -D-2Ac-Manp-(1 $\rightarrow 4$)- β -D-Manp-(1 $\rightarrow$
		H1	4.85/3.96	H4	F	$\rightarrow 4$)- β -D-2Ac-Manp-(1 $\rightarrow 4$)- β -D-2Ac-Manp-(1 $\rightarrow$
F	$\rightarrow 4$)- β -D-3Ac-Manp -(1 $\rightarrow$	H1	4.75/3.73	H4	A	$\rightarrow 4$)- β -D-2Ac-Manp-(1 $\rightarrow 4$)- β -D-Manp-(1 $\rightarrow$
		H1	4.75/3.82	H4	C	$\rightarrow 4$)- β -D-2Ac-Manp-(1 $\rightarrow 4$)- β -D-2Ac-Manp-(1 $\rightarrow$

Note: Ac, Acetyl group.

Through comprehensive analysis of glycosidic linkage patterns, monosaccharide composition, and methylation data, the molecular architecture of the repeating unit in BsPsIII1-1 was elucidated as $\rightarrow \{4\}\text{-}\beta\text{-D-Glcp-(1)}_x \rightarrow \{4\}\text{-}\beta\text{-D-Manp-(1)}_n \rightarrow \{4\}\text{-}\beta\text{-D-Glcp-(1)}_y \rightarrow \{4\}\text{-}\beta\text{-D-2Ac-Manp-(1}\rightarrow 4\text{)-}\beta\text{-D-3Ac-Manp-(1}\rightarrow 4\text{)-}\beta\text{-D-2Ac-Manp-(1}\rightarrow \{4\}\text{-}\beta\text{-D-Manp-(1)}_m \rightarrow \{4\}\text{-}\beta\text{-D-3Ac-Manp-(1}\rightarrow \{4\}\text{-}\beta\text{-D-Manp-(1)}_i \rightarrow$. The structural determination was further supported by quantitative ¹H NMR spectroscopy, in which we analyzed the peak areas, which were intrinsically proportional to the number of corresponding hydrogen atoms. By integrating the peak area data with the molar ratios of constituent monosaccharides and the average Mw, the stoichiometric relationships were established as $x + y = 8$ and $n + m + i = 4$. The polysaccharide structures were rendered in SNFG format using CSDB/SNFG Glycan Builder^[50], as shown in Fig.3G.

3.7 Morphological Characteristics with Direct Observation and SEM

To investigate the higher-order structural features of BsPsIII1-1, we employed Congo red binding assays and morphological characterization techniques. The Congo red binding assay serves as a robust analytical method for detecting conformational changes in polysaccharide chain morphology [51]. The interaction between Congo red and triple-helical polysaccharides induced a characteristic red shift in the $\lambda_{\max}$, which exhibited concentration-dependent behavior with respect to NaOH. Within the NaOH concentration range of 0.0–0.5 M, $\lambda_{\max}$ demonstrated an initial significant increase, followed by a marked decrease beyond 0.3 M, indicative of the distinctive conformational transition associated with triple-helical structures. As shown in Fig.4A, the BsPsIII1-1-Congo red complex exhibited a progressive increase in $\lambda_{\max}$ with increasing NaOH concentrations (0.0–0.3 M), subsequently decreasing at concentrations above 0.3 M. Control experiments conducted in the absence of BsPsIII1-1 showed no observable red shift, thereby confirming the presence of a triple-helical conformation in BsPsIII1-1.

Morphological characterization was performed through macroscopic observation and SEM analysis. Following lyophilization, BsPsIII1-1 presented as a white to off-white, cotton-like material with distinct glossy regions, as illustrated in Fig.4B. The material exhibited physical transformation into a powdered form upon mechanical compression. SEM analysis (Figs.4C–4E) revealed that at 1,000 $\times$ magnification, BsPsIII1-1 displayed a heterogeneous morphology consisting of both strip-like structures and irregularly shaped flat surfaces with occasional surface corrugations interspersed with smooth, featureless areas. At 5,000 $\times$ magnification, these structures exhibited increased surface roughness characterized by shallow wrinkles and numerous circular microindentations (Fig.4D). Further magnification to 10,000 $\times$ revealed additional microstructural features, including fine surface cracks superimposed on the previously observed morphological characteristics (Fig.4E).

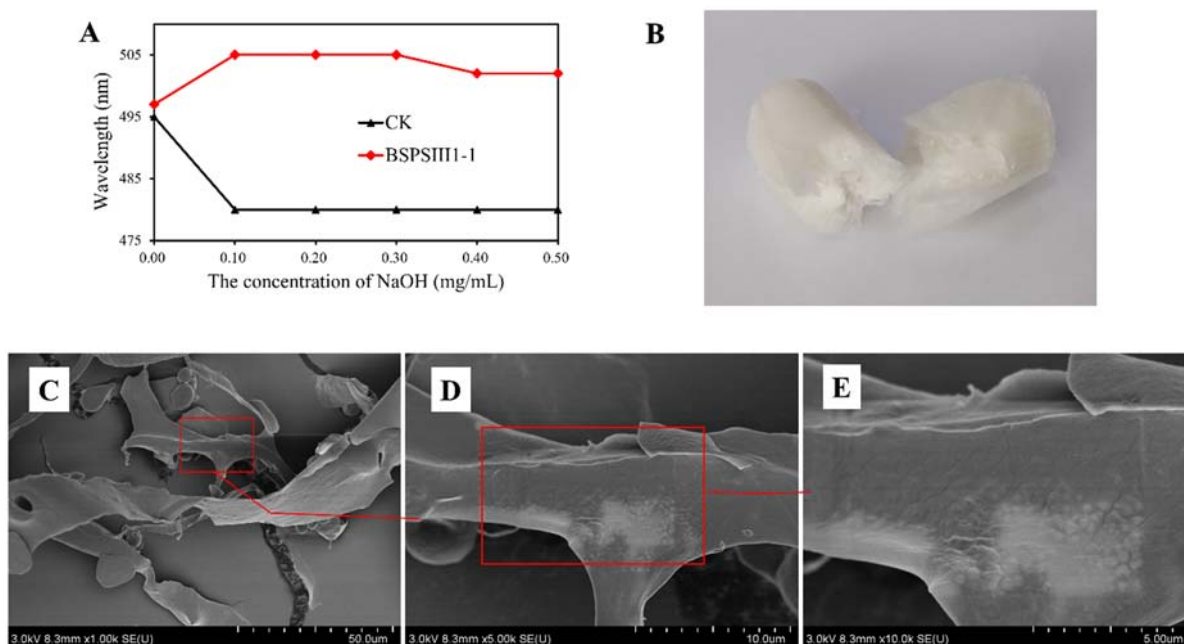


Fig.4. Congo red binding assay and morphological characterization of BsPsIII1-1. (A) maximum absorption wavelengths ($\lambda_{\max}$) of BsPsIII1-1-Congo red complexes at NaOH concentrations ranging from 0.0 to 0.5 M. (B) photographic images of the freeze-drying sample of BsPsIII1-1. Scanning electron micrographs of freeze-dried BsPsIII1-1 at magnifications of (C) 1.0 k $\times$, (D) 5.0 k $\times$, and (E) 10.0 k $\times$.

3.8 Evaluation of the hepatoprotective activities of BsPsIII1-1 *in vitro*

Using LX2 cells, we first confirmed BsPsIII1-1 (0.10-4.00 mg/mL) exhibited no cytotoxicity ($> 90\%$ viability, $P > 0.05$; Fig.5A). WB analysis subsequently revealed that 2.00 mg/mL BsPsIII1-1 significantly reduced protein levels of fibrosis markers COL-1 and α -SMA ($P < 0.01$ and $P < 0.05$, respectively), indicating potent amelioration of hepatic fibrosis, whereas 0.50 mg/mL showed no significant effect (Figs.5B-D). This dose-dependent response aligns with findings on the RG-I pectin-like polysaccharide from *Rosa chinensis* [52]. These results demonstrated that BsPsIII1-1 at 2.00 mg/mL exerts significant antifibrotic effects *in vitro* by downregulating key ECM proteins; however, the underlying mechanisms and *in vivo* efficacy required further investigation.

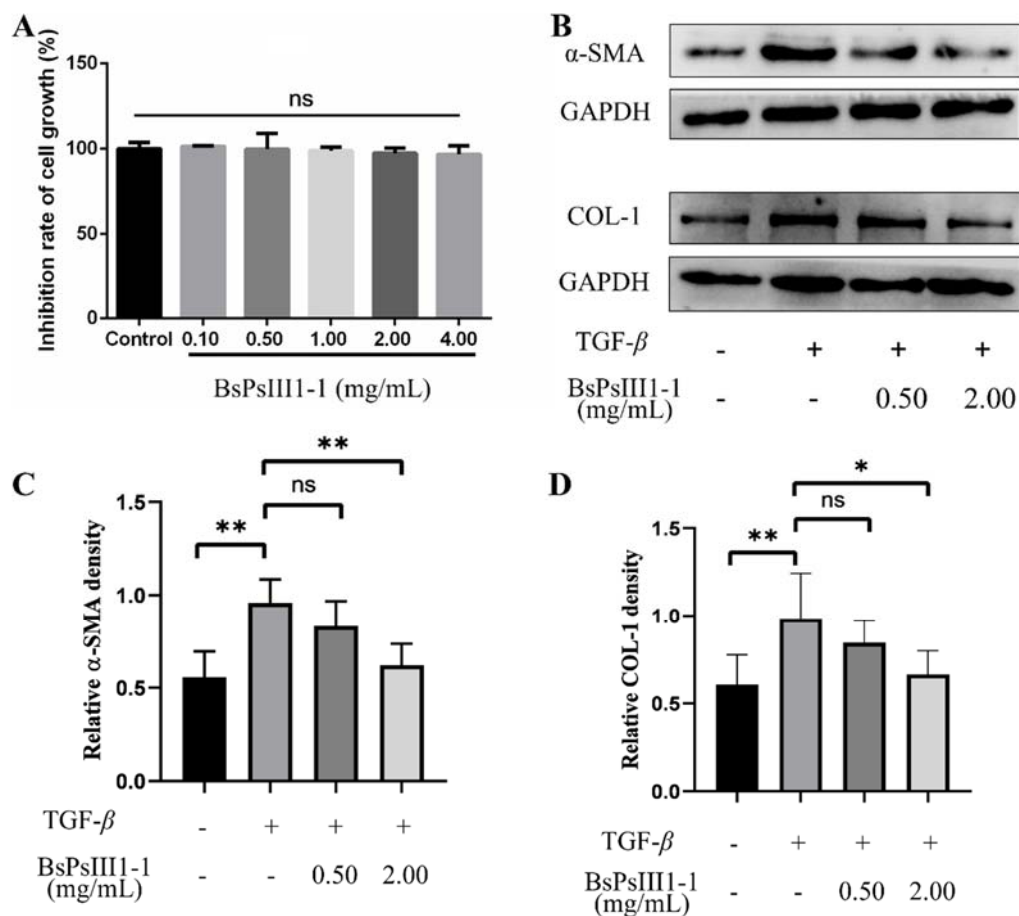


Fig.5. Effect of BsPsIII1-1 on hepatic stellate cell proliferation and activation. (A) inhibitory effect of BsPsIII1-1 on LX-2 cell growth at various concentrations (0.1, 0.5, 1.0, 2.0, and 4.0 mg/mL). (B) representative WB images showing the protein expression levels of COL-1 and α -SMA in LX-2 cells. (C and D) quantitative densitometric analysis of α -SMA (C) and COL-1 (D) protein expression normalized to the internal control. Data are presented as mean $\pm$ SD from 5 independent experiments. * $P < 0.05$ and ** $P < 0.01$. ns: not significant.

3.9 Evaluation of the Hepatoprotective Activities of BsPsIII1-1 *In Vivo*

3.9.1 Effect of BsPsIII1-1 on ALT/AST Levels and Hepatic Morphology

The hepatoprotective effects of BsPsIII1-1 (100 and 400 mg/kg) against CCl_4 -induced hepatic fibrosis were assessed, with silymarin (50 mg/kg) used as a positive control, as shown in Fig.6A. We systematically evaluated morphological alterations in hepatic tissues through visual inspection. The control group exhibited hepatic parenchyma with smooth surface architecture, uniform reddish coloration, distinct lobular margins,

and preserved tissue elasticity. By contrast, the model group demonstrated significant pathological changes, including surface irregularities, granular texture, and reduced tissue elasticity. Silymarin restored morphology to near-normal levels. Administration of BsPsIII1-1 demonstrated a dose-dependent amelioration of morphological parameters compared with the model group, indicating therapeutic efficacy in hepatic tissue preservation (Fig.6B).

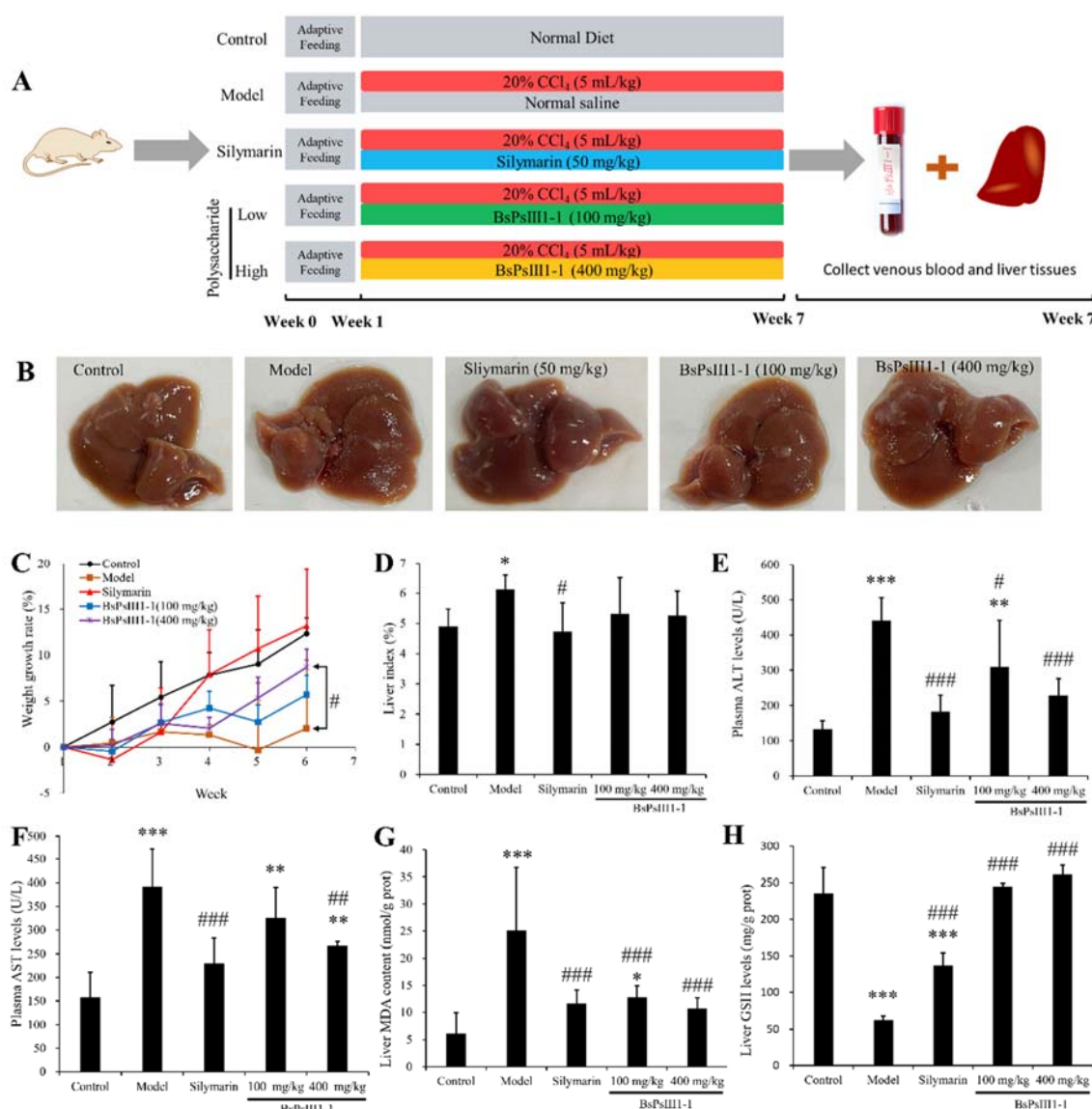


Fig.6. Protective effects of BsPsIII1-1 against CCl₄-induced liver injury in C57BL/6J mice. (A) schematic illustration of the animal experimental design. (B) gross morphology of liver tissues. (C) body weight and (D) liver index (liver weight/body weight ratio). Serum levels of (E) ALT and (F) AST. Hepatic contents of oxidative stress markers (G) MDA and (H) GSH. Data are presented as mean±SD, (n ≥ 4). # *P* < 0.05, ##: *P* < 0.01 and ###: *P* < 0.001 vs. model control. *: *P* < 0.05, **: *P* < 0.01 and ***: *P* < 0.001 vs. control.

The experimental findings demonstrated that administration of a high dose of BsPsIII1-1 (400 mg/kg) significantly attenuated CCl₄-induced body weight reduction (*P* < 0.05, Fig.6C). However, comparative analysis of liver indices revealed no statistically significant differences between BsPsIII1-1-treated groups and the model group (Fig.6D), which was potentially attributable to incomplete resolution of hepatic enlargement during the experimental period. To further evaluate hepatoprotective effects, we analyzed additional biomarkers of hepatic injury. Given that serum liver enzyme levels serve as sensitive indicators of

hepatocellular damage and hepatotoxicity^[53], we collected serum samples from all experimental groups for quantification of ALT and AST levels. Compared with the control group, the model group exhibited significant elevations in both enzymes ($P < 0.01$), with ALT levels increasing approximately 2.33-fold and AST levels increasing 1.5-fold, thereby confirming successful establishment of the hepatotoxicity model. By contrast, the high-dose BsPsIII1-1 group demonstrated significant reductions in CCl₄-induced elevations of both ALT (Fig.6E) and AST (Fig.6F) levels ($P < 0.01$), with ALT decreasing by approximately 50% and AST by approximately 32%. Notably, we observed no statistically significant differences in ALT and AST levels between the positive control group (silymarin) and the high-dose BsPsIII1-1 group. These results collectively suggested that BsPsIII1-1 exerted significant hepatoprotective effects against CCl₄-induced hepatocellular damage.

3.9.2 Effect of BsPsIII1-1 on enzymatic and nonenzymatic antioxidant

As depicted in Fig.6G, the model group exhibited a significant elevation in MDA levels ($P < 0.01$) relative to the control group, demonstrating that CCl₄ administration induced pronounced lipid peroxidation in C57BL/6J mice. Oral administration of BsPsIII1-1 at both low (100 mg/kg) and high (400 mg/kg) doses markedly attenuated hepatic lipid peroxidation, reducing MDA levels by 50% and 58%, respectively. The MDA levels in the BsPsIII1-1-treated groups were comparable to those in the positive control group. Similarly, Fig.6H revealed a significant depletion in GSH levels in the model group compared with the control group. Treatment with BsPsIII1-1 at both doses resulted in a substantial restoration of hepatic GSH levels ($P < 0.001$), which surpassed those observed in the control group.

3.9.3 Histopathological analysis of liver tissues

As illustrated in Fig.7A, the control group demonstrated normal hepatic histology, characterized by uniformly distributed hepatocytes with distinct nuclei and well-preserved tissue organization. By contrast, CCl₄-treated mice exhibited substantial hepatic damage, manifested by hepatocyte enlargement, cytoplasmic vacuolation (ballooning degeneration), cellular shrinkage, fragmentation, and dissolution indicative of cell death in addition to lipid accumulation, extensive inflammatory infiltration, and loss of cellular boundaries. Administration of BsPsIII1-1 at varying doses promoted hepatic recovery, evidenced by restoration of cellular borders, nuclear visibility, and reduced cellular swelling. Notably, the 400 mg/kg dose exhibited therapeutic effects comparable to the positive control. These observations indicated that treatment with BsPsIII1-1 significantly mitigated CCl₄-induced hepatic injury.

The present study employed Masson's trichrome and sirius red staining techniques to systematically evaluate collagen fiber alterations in hepatic tissue. Masson's trichrome staining specifically demarcated collagen fibers through aniline blue staining while contrasting with red or purplish-red cytoplasmic and nuclear components. Histopathological examination demonstrated that liver tissue from the model group exhibited extensive blue-stained collagen fiber deposition, indicative of pronounced fibrotic transformation (Fig.7B). This pathological manifestation was characterized by the accumulation of collagen bundles and the formation of fibrous septa. By contrast, BsPsIII1-1-treated groups displayed well-organized hepatocyte

architecture with diminished fibrous septa formation. In particular, the high-dose treatment group showed marked improvements in cellular morphology, including reduced inflammatory cell infiltration, attenuated collagen fiber deposition, and diminished fibrotic hyperplasia, collectively contributing to the restoration of normal hepatic architecture. Semiquantitative analysis confirmed significant, dose-dependent reduction of fibrosis in BsPsIII1-1-treated groups ($P < 0.001$, Fig.7D), with no significant difference between high-dose BsPsIII1-1 and silymarin.

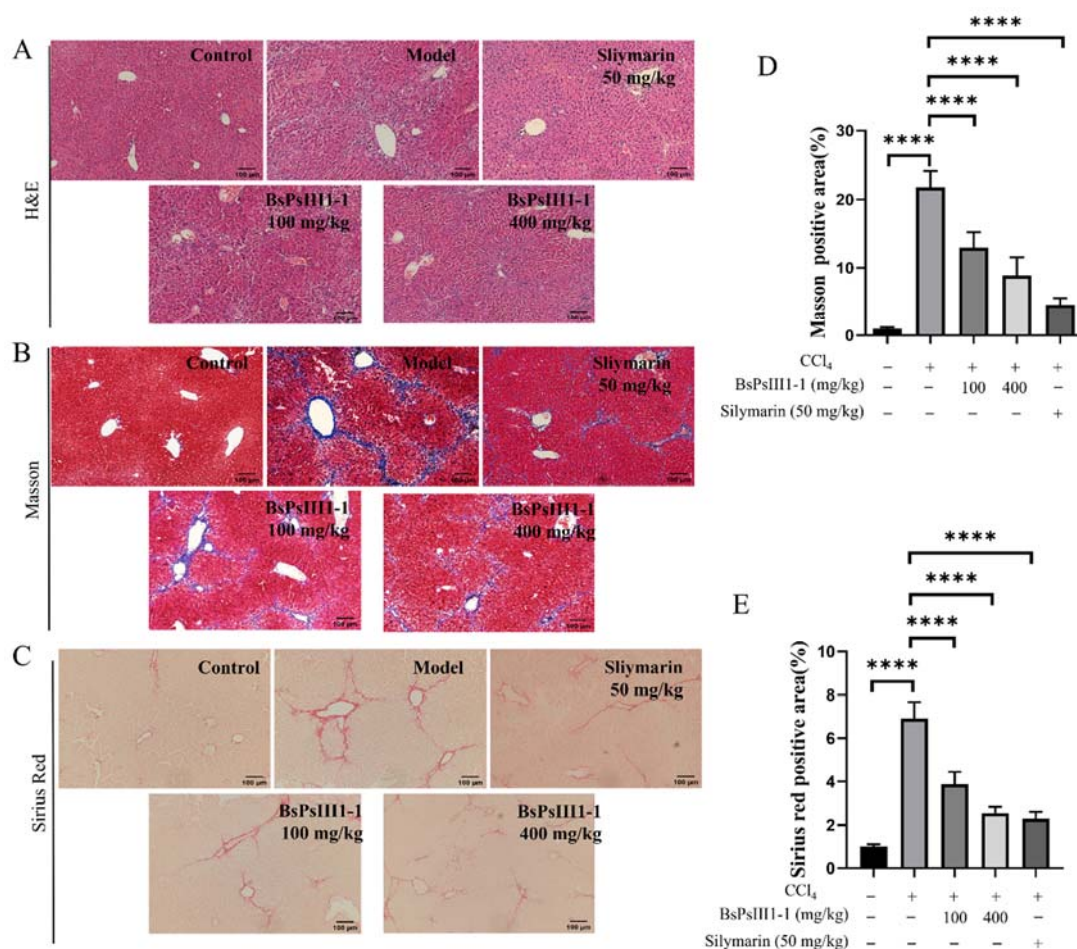


Fig.7. Histopathological evaluation of BsPsIII1-1 on CCl₄-induced liver fibrosis in mice. (A-C) representative liver histology images stained with HE (A), Masson's trichrome (B), and sirius red (C). (D and E) quantification of collagen area fraction from Masson's trichrome (D) and sirius red (E) staining analyzed by ImageJ. Data are presented as mean±SD, (n=5). ****: $P < 0.001$.

Sirius red staining selectively stains collagen fibers in a striking red or pink hue due to its specific affinity for collagen whereas noncollagenous tissue components appear as a contrasting pale-yellow background. This staining revealed a pronounced increase in collagen fiber deposition, and the formation of fibrous septa was evident in CCl₄-induced mouse liver tissue (Fig.7C). However, treatment with BsPsIII1-1 dose-dependently mitigated collagen accumulation, with the high-dose group exhibiting a substantial reduction, nearly equivalent to the efficacy of positive control. Quantitative analysis of sirius red-stained sections revealed a significantly elevated fibrosis level in the model group compared with the control ($P < 0.001$). Following BsPsIII1-1 administration, hepatic fibrosis was attenuated across all treatment groups, with the high-dose group demonstrating a significant 63% reduction ($P < 0.001$), comparable to the fibrosis level observed in the

positive control (Fig.7E). Collectively, the data from Masson and sirius red staining analyses indicated that BsPsIII1-1 significantly attenuated collagen deposition and ameliorates hepatic fibrosis.

3.10 Effects of BsPsIII1-1 on α -SMA and COL-1 expression

Immunohistochemical analysis revealed a marked elevation in the α -SMA-positive area within the livers of CCl₄-treated mice relative to normal controls (Fig.8A). Administration of BsPsIII1-1 elicited a dose-dependent attenuation of α -SMA-positive regions (Fig.8B), with semiquantitative analysis substantiating a significant reduction in α -SMA expression ($P < 0.001$). These observations were further validated by WB analysis, which demonstrated a pronounced decrease in α -SMA expression in liver tissues treated with silymarin and varying concentrations of BsPsIII1-1 compared with the model group ($P < 0.05$) (Figs.8E and 8G). Collectively, these findings suggested that BsPsIII1-1 ameliorated liver fibrosis through the suppression of α -SMA expression.

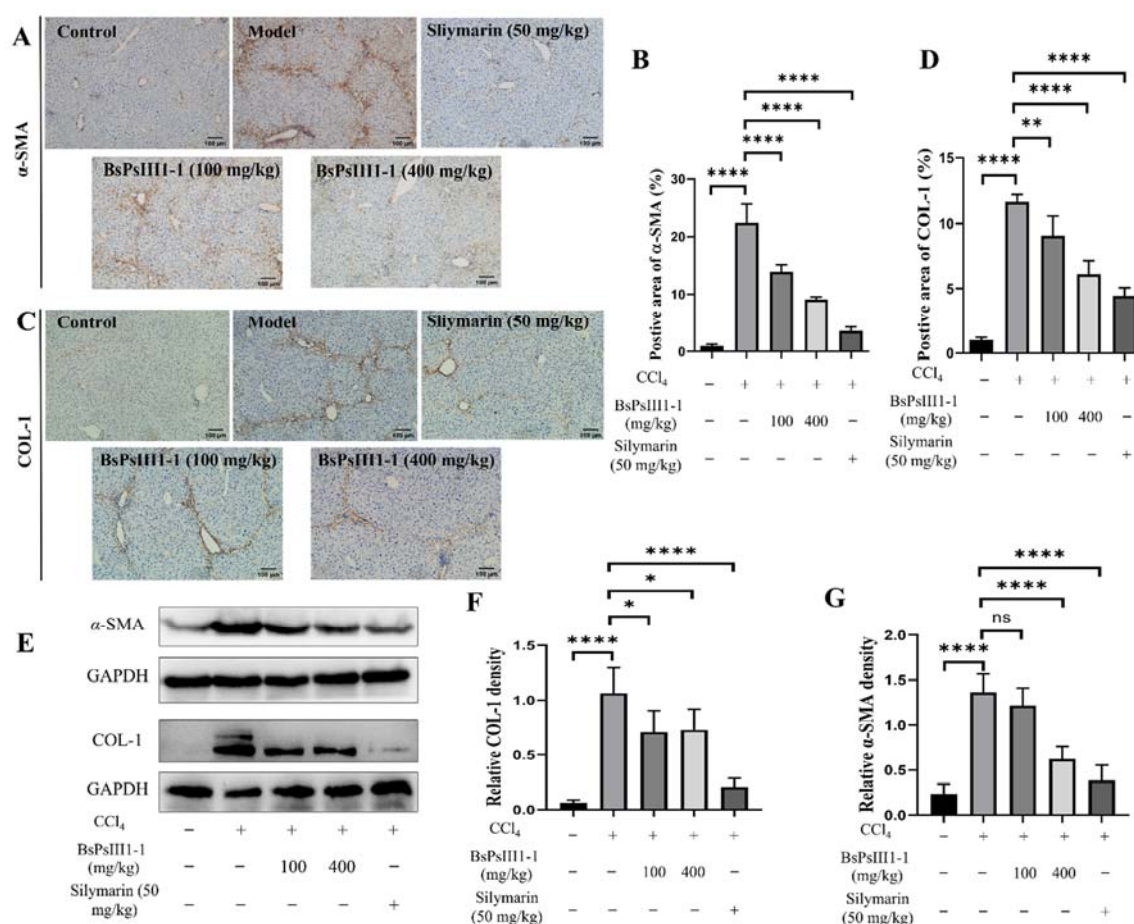


Fig.8. Effects of BsPsIII1-1 on hepatic stellate cell activation and collagen deposition in fibrotic mouse livers. representative immunohistochemical staining images showing the expression of (A and B) α -SMA and (C and D) COL-1 in liver tissues. (E) representative WB bands for α -SMA, COL-1. Semi-quantitative analysis of (F) COL-1 and (G) α -SMA protein expression normalized to GAPDH. Data are presented as mean $\pm$ SD ($n \geq 3$). *: $P < 0.05$, **: $P < 0.01$ and ***: $P < 0.001$. ns: not significant.

In control mice, COL-1 expression was limited to portal regions (Fig.8C). However, CCl₄-treated mice displayed widespread COL-1 deposition, forming star-shaped diffuse patterns around sinusoids and central veins. Both BsPsIII1-1 and silymarin significantly decreased COL-1-positive areas (Fig.8C). Semiquantitative analysis demonstrated that BsPsIII1-1 inhibited COL-1 in a dose-dependent manner, with

notable reductions compared with the model group ($P < 0.01$) (Fig.8D). The high-dose BsPsIII1-1 group showed particularly strong effects ($P < 0.001$), matching the efficacy of silymarin. WB analysis confirmed these findings, revealing substantial declines in COL-1 expression after silymarin and high-dose BsPsIII1-1 treatment ($P < 0.001$) (Figs.8E and 8F).

Together, these results indicated that BsPsIII1-1 alleviated liver fibrosis by suppressing α -SMA and COL-1 expression.

3.11 Modulatory effects of BsPsIII1-1 on the TGF- β 1/SMAD2/4/7 signaling pathway

To elucidate the molecular mechanisms underlying this signaling pathway, we systematically quantified protein expression profiles in both serum and liver tissue samples. Serum TGF- β 1 concentrations were determined using ELISA, and the expression levels of TGF- β 1, SMAD2, *p*-SMAD2, SMAD4, and SMAD7 in liver tissue were analyzed using WB (Fig.9). As demonstrated in Figs.9A-C, CCl₄ administration significantly activated the TGF- β 1 signaling pathway, as indicated by substantial increases in both serum ($P < 0.01$) and hepatic ($P < 0.001$) TGF- β 1 levels. To further investigate downstream signaling events, we examined the expression of key SMAD family proteins. CCl₄ treatment resulted in a significant elevation of *p*-SMAD2, as evidenced by the *p*-SMAD2/SMAD2 ratio ($P < 0.001$), and increased SMAD4 protein expression ($P < 0.001$). However, the expression of the inhibitory SMAD7 was markedly reduced ($P < 0.001$).

By contrast, oral administration of BsPsIII1-1 exhibited a dose-dependent suppression of the TGF- β 1/SMAD signaling pathway. High-dose BsPsIII1-1 treatment demonstrated pronounced efficacy, significantly attenuating TGF- β 1 levels in both serum ($P < 0.01$) and liver tissue ($P < 0.001$; Figs.9A-C), thereby reversing the CCl₄-induced elevation. The treatment also inhibited the downstream SMAD pathway, as evidenced by a reduction in the *p*-SMAD2/SMAD2 ratio ($P < 0.01$) and the protein expression of SMAD4 ($P < 0.001$), indicating robust suppression of canonical SMAD pathway activation. Additionally, BsPsIII1-1 upregulated the expression of the negative regulatory factor SMAD7 ($P < 0.01$), further attenuating the activation of the TGF- β 1/SMAD signaling cascade and downregulating the expression of key fibrotic markers, including α -SMA and COL-1 (Fig.9). These findings collectively identified the TGF- β 1/SMAD pathway as a critical signaling axis through which BsPsIII1-1 mediated the reversal or alleviation of CCl₄-induced hepatic fibrosis in mice, underscoring its hepatoprotective properties. A schematic representation of this mechanistic pathway is provided in Fig.9G.

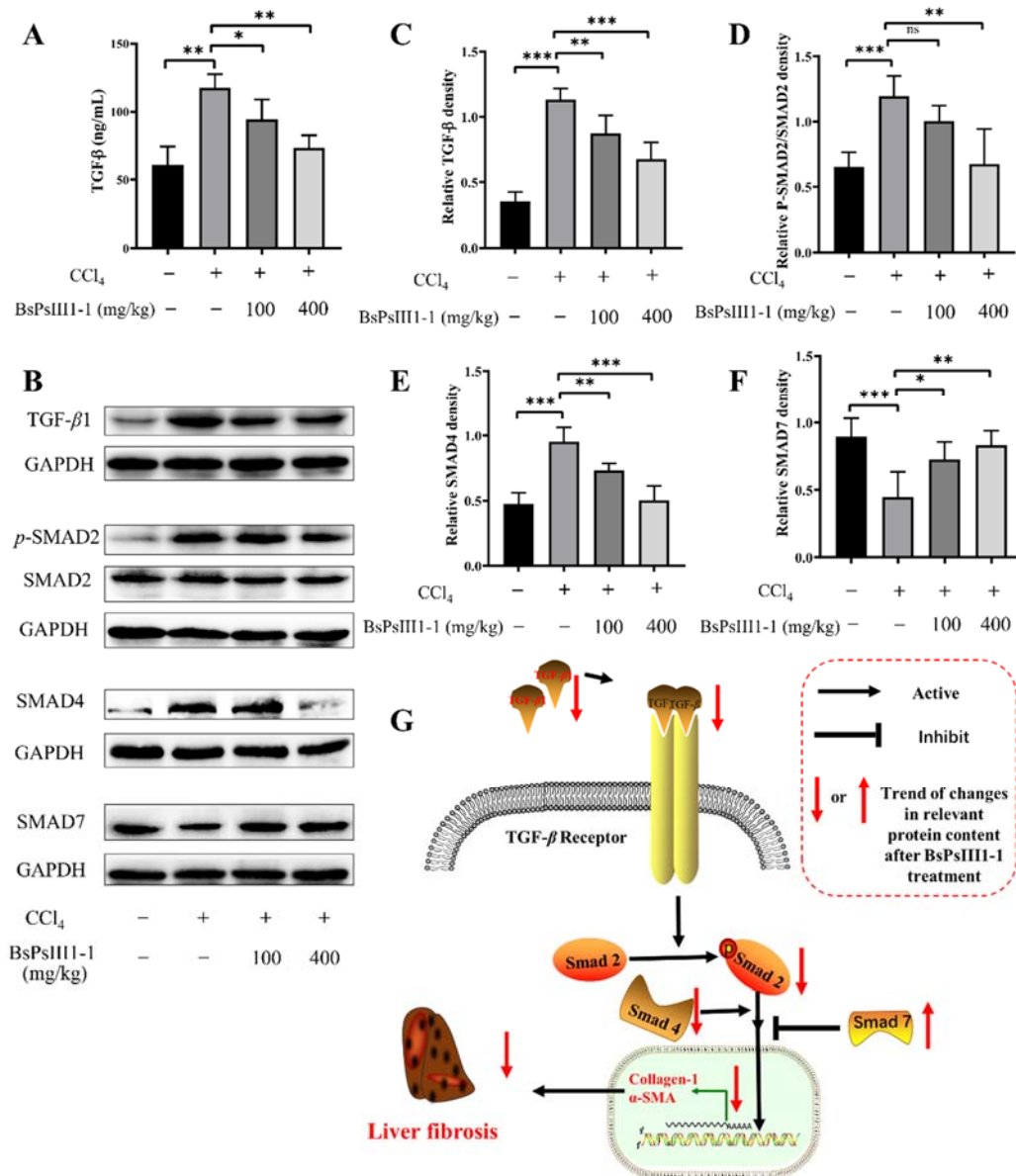


Fig.9. The expression of TGF-β1/SMAD2/4/7 signaling pathway-related proteins in C57 mice. (A) serum levels of TGF-β1 in different treatment groups. (B) WB analysis of TGF-β1/SMAD2/4/7 pathway proteins. (C-F) quantitative analysis of western blot results for key proteins in the TGF-β1/SMAD2/4/7 pathway. (G) a schematic representation of this entire process. Data are presented as mean±SD, (n=5). *: $P < 0.05$, **: $P < 0.01$, and ***: $P < 0.001$. ns: not significant.

3.13 BsPsIII1-1 Modulates Gut Microbiota

To examine alterations in gut microbiota abundance associated with BsPsIII1-1, we performed 16S rRNA sequencing on murine fecal samples. As illustrated in Fig.10A, a total of 495 OTUs were identified across all samples, with 300 OTUs in the control group, 279 OTUs in the model group, and 294 OTUs in the BsPsIII1-1 group. The control group identified 94 unique OTUs, the model group detected 68 unique OTUs, and the experimental group treated with BsPsIII1-1 intervention exhibited 88 unique OTUs. Notably, 34 OTUs retained in the control group but lost in the model group reappeared following BsPsIII1-1 intervention.

Alpha diversity analysis revealed comparable microbial richness across groups as assessed by the Chao1 index (Fig.10B). Simpson's index (higher scores reflect greater dominance and reduced diversity) demonstrated a stepwise decrease as evidenced by significantly reduced dominance in the model group compared with controls ($P < 0.05$) and a further significant reduction following BsPsIII1-1 treatment ($P <$

0.05, Fig.10C). Principal coordinate analysis (PCoA) of β -diversity further confirmed significant differences in gut microbiota composition, as evidenced by the clear separation between groups (Fig.10D).

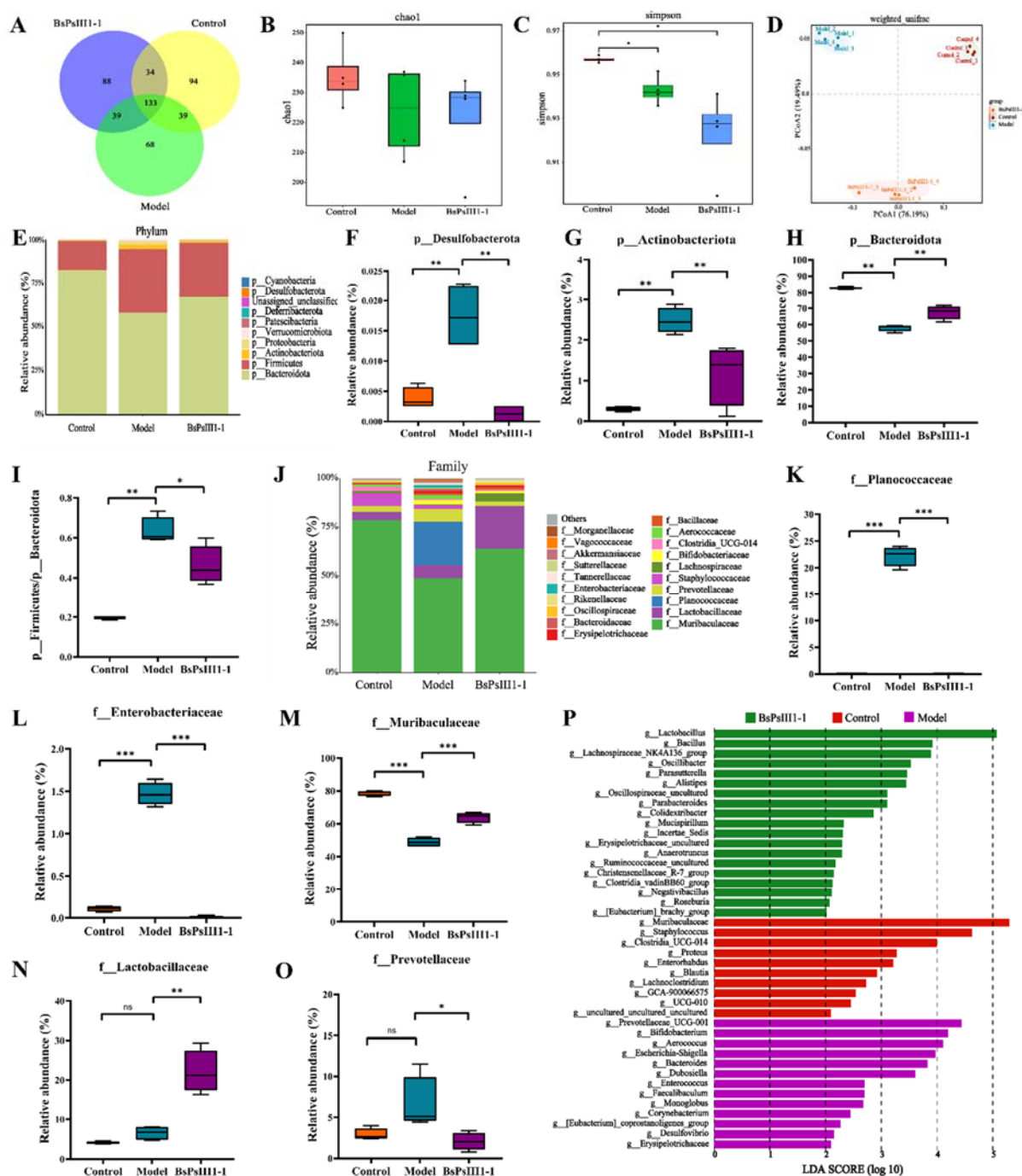


Fig.10. BsPsIII-1 modulates gut microbiota in hepatic fibrosis. (A) Venn diagram showing shared and unique OTUs. (B-C) alpha diversity (Chao1 and Simpson). (D) PCoA plot (weighted UniFrac). (E) Phylum-level relative abundance. (F-H) Desulfobacterota, Actinobacteriota, and Bacteroidota abundances. (I) F/B ratio. (J) family-level composition. (K-O) abundances of Planococcaceae, Enterobacteriaceae, Muribaculaceae, Lactobacillaceae, and Prevotellaceae. (P) LefSe analysis identifying discriminative genera (LDA > 4.0). Data are presented as mean±SD, (n=4). *: $P < 0.05$, **: $P < 0.01$, and ***: $P < 0.001$. ns: not significant.

At the phylum level, the model group displayed significant enrichment of p_Desulfobacterota (sulfate-reducing bacteria) and p_Actinobacteriota, concomitant with depletion of p_Bacteroidota ($P < 0.05$, Figs.10E-H). BsPsIII1-1 treatment reversed these alterations, significantly reducing p_Desulfobacterota and p_Actinobacteriota while restoring p_Bacteroidota levels ($P < 0.05$). Additionally, treatment with BsPsIII1-1

returned the elevated p_Firmicutes/ p_Bacteroidetes ratio (F/B ratio) in the model group to normal levels ($P < 0.05$, Fig.10I).

Family-level analysis (Wilcoxon rank-sum test) showed that the model group was characterized by increased f_Planococcaceae and f_Enterobacteriaceae alongside decreased f_Muribaculaceae ($P < 0.05$, Figs.10K-M). BsPsIII1-1 reversed these changes by suppressing f_Planococcaceae and f_Enterobacteriaceae and restoring f_Muribaculaceae ($P < 0.05$). Beyond reversing model-induced dysbiosis, BsPsIII1-1 significantly enriched f_Lactobacillaceae and reduced f_Prevotellaceae, despite no significant differences in these taxa between control and model groups ($P < 0.05$, Figs.10N-O).

Consistently, LEfSe analysis ($LDA > 4.0$, $P < 0.05$) at the genus level revealed g_Planococcaceae-UCG-001 as dominant biomarkers in the model group whereas g_Lactobacillus was the primary biomarker in the BsPsIII1-1 group (Fig.10P). BsPsIII1-1 induced a compositional shift of the gut microbiota from a g_Planococcaceae-characterized state to a g_Lactobacillus-dominated configuration. This finding was consistent with the aforementioned family-level analysis results. Collectively, these data demonstrated that CCl₄-induced hepatic fibrosis was associated with significant restructuring of the gut microbiota, characterized by expansion of potential pro-inflammatory taxa (f_Enterobacteriaceae, f_Desulfobacterota) and depletion of commensal fiber-fermenting populations (f_Muribaculaceae), which BsPsIII1-1 substantially reversed. Given that these compositional shifts particularly affected taxa involved in SCFAs biosynthesis, we subsequently quantified fecal SCFAs levels to assess the functional metabolic consequences of these alterations.

3.14 BsPsIII1-1 Modulates Gut Microbiota-Derived SCFAs

Quantitative assessment revealed a substantial reduction in the concentrations of multiple SCFAs in the CCl₄-induced model group compared with the control group, including acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, isovaleric acid, hexanoic acid, and 4-methylvaleric acid ($P < 0.001$; Figs.11A-B). To assess the therapeutic efficacy of BsPsIII1-1, we examined its effects on the SCFAs profile at a dosage of 400 mg/kg, which exhibited a differential regulatory pattern across various SCFAs (Figs.11A-B). Among the minor SCFAs components, the BsPsIII1-1 treatment group showed further reduction in butyric acid and valeric acid levels compared with the model group whereas isovaleric acid and 4-methylvaleric acid were significantly restored ($P < 0.001$; Fig. 11A). Notably, hexanoic acid levels remained unaltered following treatment (Fig.11A). Analysis of major SCFAs indicated a significant upregulation of acetic acid and propionic acid concentrations after BsPsIII1-1 administration ($P < 0.01$) whereas isobutyric acid levels remained unchanged (Fig.11B). Collectively, the total SCFAs content in the BsPsIII1-1 intervention group showed a significant increase compared with the model group ($P < 0.01$; Fig.11C), suggesting a comprehensive enhancement of SCFAs metabolic pathways.

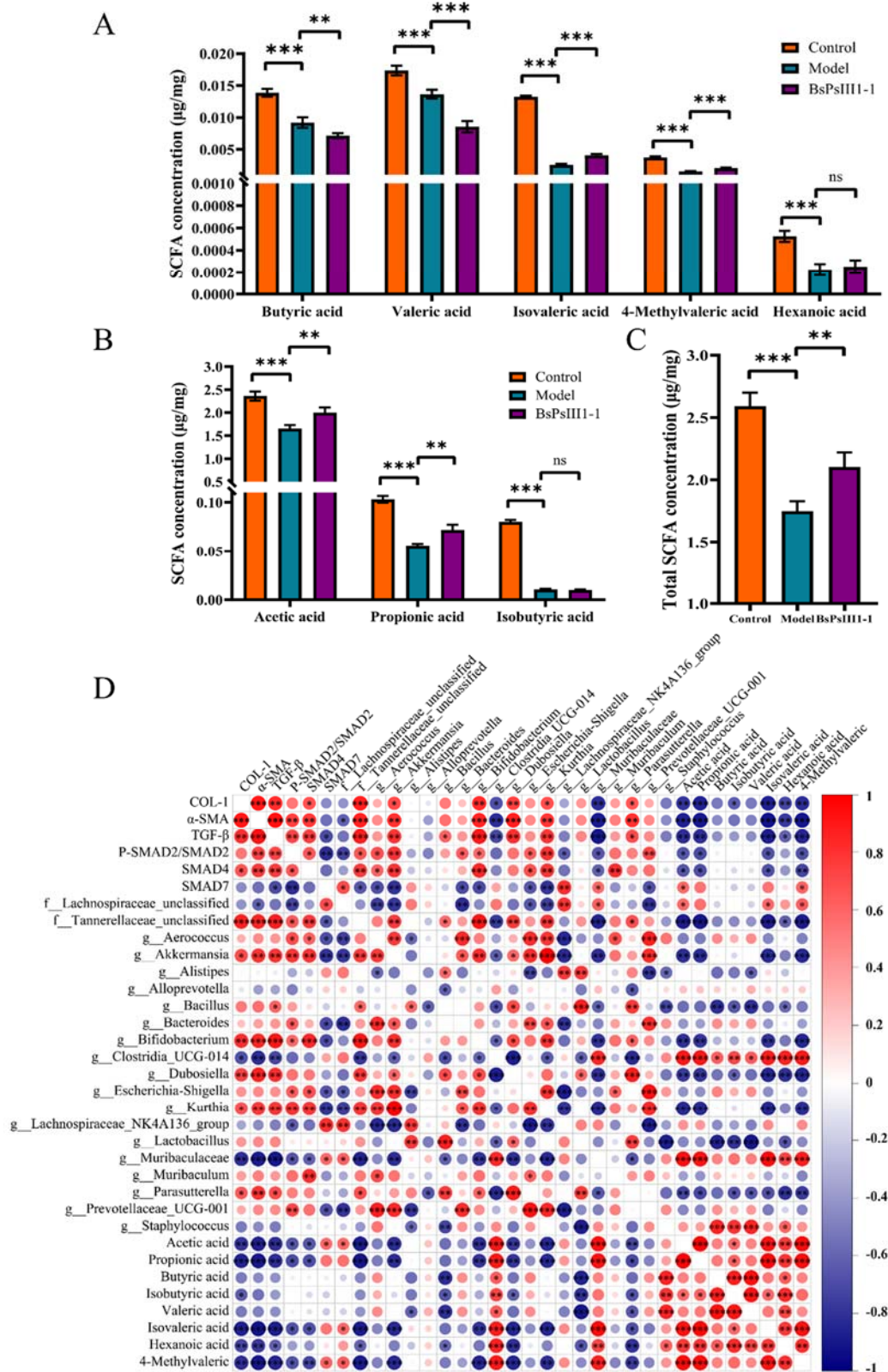


Fig.11. BsPsIII1-1 modulates fecal SCFAs metabolism and establishes a microbiota-SCFAs-fibrosis axis. (A and B) concentrations of (A) high-abundance SCFAs (acetate, propionate, and butyrate) and (B) low-abundance/branched-chain SCFAs (isobutyrate, valerate, isovalerate, hexanoate, and 4-methylvalerate). (C) total SCFAs content. (D) Spearman correlation analysis among differential bacterial genera, SCFAs, and hepatic fibrosis markers (COL-1, α -SMA, TGF- β , and SMAD7). Data are presented as mean $\pm$ SD, (n=4). *: $P < 0.05$, **: $P < 0.01$, and ***: $P < 0.001$. ns: not significant.

Given the concurrent remodeling of the gut microbiota and the alteration of fecal SCFAs profiles following BsPsIII1-1 intervention, we next investigated functional linkages among bacterial genera, SCFAs

metabolism, and hepatic fibrosis markers. Spearman correlation analysis among the top 20 differential genera, 8 SCFAs, and SMAD pathway proteins revealed distinct association patterns (Fig.11D). *g_Clostridia* UCG-014 and *g_Muribaculaceae* abundance was positively correlated with acetate acids, propionate acids, isovalerate acids, and 4-methylvaleric acid levels ($P < 0.001$) whereas *f_Tannerellaceae-unclassified* showed negative correlations with these metabolites ($P < 0.001$). Furthermore, SCFAs concentrations displayed significant inverse relationships with fibrosis markers. Acetate acids, propionate acids, and isovalerate acids were negatively associated with COL-1, α -SMA, TGF- β , P-SMAD2/SMAD2 and SMAD4 ($P < 0.01$) but positively correlated with the inhibitory regulator SMAD7 ($P < 0.05$). After intervention with BsPsIII1-1, intestinal microbiota analysis showed a significant increase in Muribaculaceae, promoting the increase of SCFAs and thereby inhibiting the activation of the SMAD signaling pathway and consequently reducing the expression of liver fibrosis markers COL-1 and α -SMA.

4. Discussion

In this study, we identified a novel neutral polysaccharide, BsPsIII1-1, was identified from three-year-old *B. striata* tubers collected in Zunyi, a renowned medicinal herb-producing region in southwest China. This discovery was achieved through systematic isolation, purification, and structural elucidation. Structural analysis revealed a unique monosaccharide composition, with a Man to Glc molar ratio of 1:1, consistent with previous analyses of crude polysaccharides from this material [12]. Although some earlier studies had reported *B. striata* polysaccharides with a similar Man:Glc ratio, their purified homogeneous polysaccharides contained $\rightarrow 4$)- α -D-Glcp (50.15%), $\rightarrow 4$)- α -D-Manp (22.61%), and $\rightarrow 3$)- α -D-Manp (25.72%), differing significantly from the structure of the polysaccharide we describe here [54]. Additionally, BsPsIII1-1 contained a minor amount of Gal, aligning with findings by Ji *et al.* [8], who reported that BsPs were primarily composed of Glc and Man, with Gal present in only two BsPs. We proposed that this might indicate environmental regulation of plant cell polysaccharide biosynthesis [55], suggesting that Gal modification was not essential for primary polysaccharide biosynthesis but rather represented a secondary modification triggered by environmental factors.

From a molecular standpoint, the Mw of BsPsIII1-1, approximately 74.8 kDa, was a crucial parameter. Numerous studies had shown that Mw significantly affects polysaccharide hydration behavior, rheological properties, and biological activities [56, 57]. Notably, natural bioactive polysaccharides with similar molecular weights had been documented. For example, Su *et al.* [43] found that enzymatic hydrolysis of *Hericium erinaceus* polysaccharide HEP (initial Mw 7.5×10^4 kDa) produced a 35 kDa fragment, FHPEP, which demonstrated enhanced antioxidant activity compared with the native polymer. These findings suggested that BsPsIII1-1 might also have biologically relevant activity meriting further investigation. Preliminary studies on the crude polysaccharide indicated antihepatic fibrosis activity. Consequently, this study focused on the antihepatic fibrosis effects of the purified polysaccharide BsPsIII1-1 to explore its functional properties in greater detail.

To assess whether BsPsIII1-1 had antihepatic fibrosis activity, we first evaluated its effects on LX2 cells. An SRB assay showed that even the highest concentration tested (4 mg/mL) did not inhibit LX2 cells growth, indicating cellular safety and supporting previous reports that BsPs are nontoxic [58]. Using two intermediate concentrations, we then demonstrated that BsPsIII1-1 suppressed α -SMA and COL-1 expression in TGF- β 1-stimulated LX2 cells in a dose-dependent manner, consistent with prior findings for low-Mw BsPs or oligosaccharides [22, 23]. Given the structural heterogeneity of polysaccharides, variations in monosaccharide composition, glycosidic linkages, and Mw, we proceeded to further investigate the anti-hepatic fibrotic properties of this polysaccharide.

CCl₄ is a well-characterized hepatotoxin that produces acute liver injury in murine models, presenting with hepatomegaly, fibrosis, and body weight loss [59]. Using a CCl₄-induced liver fibrosis model in this study, we evaluated the hepatoprotective effects of BsPsIII1-1 by monitoring body weight, liver morphology, liver index, and serum ALT and AST levels, which are established markers of liver injury severity [60]. The results demonstrated that BsPsIII1-1 provided robust protection against hepatic damage.

Extensive research has shown that BsPs exhibit potent antioxidant properties through diverse molecular mechanisms, encompassing direct ROS scavenging [61], enhancement of SOD enzymatic activity [62], and regulation of Angiotensin II (AngII)-induced ROS generation via the NOX4/p22phox signaling pathway [21]. Given that MDA indicates hepatic oxidative damage and GSH reflects the antioxidant response [63], we systematically measured hepatic MDA and GSH concentrations in a murine model to clarify mechanisms of liver injury. The results showed that BsPsIII1-1 significantly mitigated CCl₄-induced hepatic oxidative stress. These findings not only validated previous research but also established a critical role for the ROS-scavenging capacity of BsPsIII1-1 in maintaining hepatic homeostasis and preserving normal liver function.

Histopathological evaluation using H&E, Masson's trichrome, and sirius red staining unequivocally demonstrated BsPsIII1-1's ability to attenuate fibrotic progression, supporting its potential as an antifibrotic agent. Additionally, immunohistochemical and WB analyses revealed that BsPsIII1-1 markedly downregulated the expression of fibrotic markers α -SMA and COL-1 in a CCl₄-induced murine liver fibrosis model. This reduction correlated with diminished collagen deposition, further confirming its antifibrotic efficacy. Together with other hepatoprotective effects observed in our study, these findings established BsPsIII1-1 as a novel antifibrotic polysaccharide from *B. striata*.

Previous studies identified glucomannan as the primary bioactive component of *B. striata* [12, 15]. Although the antifibrotic properties of BsPs were first reported in 2014 [20], their mechanistic details and efficacy in hepatic fibrosis remain poorly understood. Critically, structural heterogeneity among these polysaccharides leads to significant variability in their biological activities and pharmacological effects [22]. Thus, systematic mechanistic investigations are essential to fully utilize their therapeutic potential against hepatic fibrosis.

We measured TGF- β 1 concentrations in mouse serum by ELISA and in liver tissue by WB. CCl₄ administration markedly activated the TGF- β 1-signaling axis in the murine model, producing significant

increases in serum and hepatic TGF- β 1 levels. Treatment with BsPsIII1-1 reduced TGF- β 1 levels significantly in both serum and liver tissue. TGF- β 1 is a central cytokine in tissue fibrosis and cancer progression [64, 65]. In liver fibrosis, HSCs are the principal effector cells, and their activation constitutes a key initiating event. This activation is driven primarily by autocrine or paracrine TGF- β 1 signaling and produces a phenotypic switch marked by increased proliferation, fibrogenesis, and contractility [66]. Thus, the antihepatic fibrosis effects of BsPsIII1-1 were found to involve the TGF- β 1-induced pathway.

Previous studies have demonstrated that the TGF- β 1-induced intracellular SMAD signaling pathway plays a pivotal role in liver fibrosis pathogenesis [67]. The process begins when TGF- β 1 binds its serine/threonine kinase receptor, triggering phosphorylation of the downstream SMAD2/3 proteins. The *p*-SMAD2/3 then forms a complex with SMAD4, which translocates to the nucleus and functions as a transcription factor to activate the expression of profibrotic genes, including COL-1 and α -SMA. Significantly, this pathway is tightly regulated by negative feedback mechanisms, with SMAD7 playing a pivotal inhibitory role by binding to the TGF- β receptor and attenuating signal transduction, thereby preserving tissue homeostasis [68]. Therefore, we performed WB analysis on key proteins in the SMAD signaling pathway. CCl₄ treatment significantly increased *p*-SMAD2/SMAD2 ratio and SMAD4 protein levels while markedly reducing inhibitory SMAD7 expression. These molecular alterations, in conjunction with the observed upregulation of α -SMA and COL-1, collectively suggested that CCl₄ induces HSCs activation, leading to TGF- β 1 secretion, subsequent SMAD signaling activation, and ultimately, the overexpression of fibrotic proteins, culminating in severe hepatic fibrosis [69]. However, after BsPsIII1-1 treatment, the expression of these proteins showed significant reversal compared with the model group. These results confirmed that BsPsIII1-1 could exert anti-liver fibrosis effects by regulating the TGF- β 1/SMAD pathway.

Previous studies showed that gut microbiota dysbiosis contributes to hepatic fibrosis, and polysaccharides' antifibrotic effects often involve modulation of the gut microbiome [70-72]. In the present study, mice in the CCl₄-induced liver fibrosis model developed pronounced diarrhea although intervention with the BsPsIII1-1 significantly alleviated these symptoms. This finding implied that in addition to acting through the canonical TGF- β 1/SMAD signaling pathway, BsPsIII1-1 might provide therapeutic benefit by restoring gut microbiota homeostasis. To evaluate this hypothesis, we performed 16S rRNA sequencing to compare intestinal microbiota composition among the experimental groups.

The gut microbiota experiment showed that CCl₄-induced liver fibrosis was accompanied by marked gut microbiota dysbiosis, characterized by a loss of beneficial taxa and an expansion of pathogenic species that likely amplified hepatic inflammation. Specifically, CCl₄ exposure increased potentially harmful taxa, including *f_Enterobacteriaceae*, *f_Planococcaceae*, and *f_Prevotellaceae*, likely reflecting inflammation and impaired gut barrier function. By contrast, BsPsIII1-1 treatment shifted the community toward *Lactobacillus* dominance, indicating selective microbial modulation as a component of its therapeutic action. Family-level analyses supported these genus-level findings, with BsPsIII1-1 suppressing pathogenic families

(f_Enterobacteriaceae, f_Planococcaceae, and f_Prevotellaceae) while enriching beneficial families (f_Lactobacillaceae and f_Muribaculaceae) [73]. Those results were consistent with prior reports that polysaccharides exerted anti-fibrotic effects via microbiome modulation [70-72].

In addition, this study confirmed through GC-MS analysis that the total SCFAs content, in the BsPsIII1-1-treated group, particularly acetate acid and propionate acid, in the BsPsIII1-1-treated group showed a significant increase compared with the model group. Mechanistically, BsPsIII1-1 modulated the composition of the gut microbiota, thereby enhancing SCFAs production. These SCFAs subsequently entered systemic circulation, reached hepatic tissues, and directly inhibited HSC activation, ultimately attenuating fibrotic progression. This finding was consistent with previous studies demonstrating that the polysaccharides of *Auricularia auricula* directly suppressed hepatic fibrosis by the gut-liver axis and enhancing SCFA metabolism [70].

To further explore the relationships among TGF- β 1/SMAD signaling, gut microbiota, and SCFAs, we conducted Spearman correlation analyses involving the top 20 differential genera, 8 SCFAs, and SMAD pathway proteins. The abundance of the g_Muribaculaceae, which increased following treatment with BsPsIII1-1, exhibited significant positive correlations with acetate, propionate, isovalerate, and 4-methylvalerate acids. These SCFAs were significantly elevated relative to the model group after polysaccharide treatment, supporting the idea that polysaccharides promote SCFAs production via expansion of g_Muribaculaceae and thereby influence liver fibrosis [74]. Acetate, propionate, isovalerate, and 4-methylvaleric acids correlated significantly and negatively with profibrotic markers, COL-1, α -SMA, TGF- β 1, p-SMAD2/SMAD2, and SMAD4 and correlated significantly and positively with the negative regulatory factor SMAD7. These findings indicated that BsPsIII1-1 could suppress the TGF- β 1/SMAD signaling pathway by modulating the gut microbiota and increasing SCFAs levels, thereby attenuating liver fibrosis. The result further supported that polysaccharide can ameliorate liver injury by improving intestinal barrier function [73].

The structural heterogeneity of BsPs and their tissue-specific variations may contribute to their diverse anti-fibrotic mechanisms. Wang S. *et al.* [19] demonstrated that a specific BsP attenuated fibrosis in L929 cells through the suppression of TGF- β 1, COL-1, vimentin, and α -SMA expression while concurrently alleviating lung fibrosis in C57BL/6 mice via regulation of p62 and p-mTOR signaling pathways. In a parallel study, Wang Y. *et al.* [20] identified a high-Mw glucomannan (BSPb) that inhibited fibrotic processes in human mesangial cells by downregulating TGF- β RI, TGF- β RII, and α -SMA expression. Furthermore, Yue *et al.* [21] reported that BSPb exhibited antirenal fibrotic effects by reducing ROS, IL-6, and TNF- α levels *in vitro*, suppressing NOX4 upregulation, and attenuating TLR2 overexpression induced by Ang II. Notably, these studies did not investigate hepatic tissues. To date, only one low-Mw BsP has been shown to significantly mitigate CCl₄-induced hepatic fibrosis through the TLR2/TLR4-MyD88-NF- κ B signaling pathway [22]. In the present study, we provided novel evidence demonstrating that a polysaccharide BsPsIII1-1 derived from *B. striata* effectively inhibited hepatic fibrosis progression via the TGF- β 1/SMAD signaling pathway and gut

microbiota, thereby complementing the established antifibrotic properties of BsPs and advancing our understanding of their mechanistic diversity in hepatic fibrosis.

Although this study demonstrated the antifibrotic efficacy of BsPsIII1-1 and elucidated its underlying mechanisms, several critical scientific questions remain unresolved. First, regarding the mechanistic investigation, current evidence suggests that BsPs exert antifibrotic effects through multiple pathways. For example, BsPs mitigated pulmonary fibrosis by modulating the TGF- β 1, α -SMA, COL-I, and p62/mTOR pathways ^[19]. Similarly, BSPb polysaccharide ameliorated renal fibrosis by suppressing ROS, IL-6, TNF- α , and the NOX4/TLR2 signaling axis ^[21]. These findings suggest that BsPsIII1-1 might target additional pathways and molecular mechanisms that remain uncharacterized. However, SCFAs are not the only upstream regulatory factors of TGF- β 1. Whether polysaccharides directly regulate or indirectly modulate the TGF- β 1/SMAD signaling pathway through other factors remains worthy of further investigation. Future investigations should employ comprehensive omics approaches to delineate its complete regulatory network, thereby providing a holistic understanding of its multitarget synergistic mechanisms. Second, concerning the structure-activity relationship (SAR), this study only characterized the structure of a single polysaccharide, leaving the specific structural motifs responsible for its antifibrotic activity unidentified. Subsequent research should systematically explore the SAR by isolating and structurally modifying diverse *B. striata* polysaccharides, facilitating the rational design of bioactive molecules. Finally, with respect to the gut microbiota-mediated mechanism, although data indicated that BsPsIII1-1 modulated the microbiota and enhanced SCFAs production, the identification of key functional bacterial strains remains essential. Validation through experimental approaches such as bacterial strain transplantation is necessary to elucidate the precise regulatory axis of specific bacterial strain-specific metabolite-specific targets.

5. Conclusion

We isolated and characterized a novel neutral high-Mw glucomannan, BsPsIII1-1, from *B. striata* in this study and demonstrated its potent antihepatic fibrosis activity. BsPsIII1-1 operated via 2 complementary mechanisms. First, it directly suppressed hepatic fibrogenesis by inhibiting TGF- β 1 release, reducing p-SMAD2 and SMAD4, upregulating SMAD7, and downregulating the fibrotic markers α -SMA and COL-1. Second, it indirectly modulated gut microbiota composition and thereby promoted production of SCFAs through the gut-liver axis. Owing to this dual-pathway activity, BsPsIII1-1 was a promising candidate both as a therapeutic agent for hepatic fibrosis and as a functional food ingredient, with potential to expand antifibrotic interventions across organs and to strengthen botanical polysaccharide-based therapies.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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