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Research article

Structural characterization and protective effect against renal fibrosis of polysaccharide from *Ligustrum lucidum* Ait.

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Abstract

Ethnopharmacological relevance: Fructus Ligustri Lucidi (FLL), the fruit of *Ligustrum lucidum* Ait., is a traditional Chinese medicine that has been used for tonifying the kidney and liver for decades.

Aim of the study: This study aimed to explore and identify polysaccharides from FLL and elucidate its protective effect against renal fibrosis.

Materials and methods: Polysaccharides were extracted and isolated from FLL. The purified fraction was identified by serial phytochemical work, such as gel-permeation chromatography, ion chromatography, gas chromatography-mass spectrometry, and nuclear magnetic resonance. Mice with unilateral ureteral obstruction (UUO) were applied as a renal fibrosis model. The male UUO mice were pretreated with heteropolysaccharide (Poly) 1 week prior to surgery and continuously treated for 7 days after the operation. Renal fibrosis was assessed by Periodic Acid-Schiff (PAS) staining and Masson's trichrome staining in paraffin-embedded slides. The murine mesangial cells SV40-MES13 upon angiotensin II (Ang II) treatment were developed as an *in vitro* fibrotic model. The cells were treated by Poly in the presence of Ang II. Molecular expression was detected by RT-PCR, immunoblotting, and immunofluorescence staining.

Results: We identified a heteropolysaccharide composed of arabinose and galactose (molar ratio, 0.73:0.27) with a predicted chemical structure characterized by a backbone composed of 1,5-α-Ara*f*, 1,3,5-α-Ara*f*, 1,6-α-Gal*p*, and 1,3,6-β-Gal*p* and side chains comprised of T-α-Ara*f*, T-α-Ara*p*, and 1,3-α-Ara*f*. Pretreatment of UUO

mice with Poly effectively alleviated glomerulosclerosis and tubulointerstitial fibrosis. Moreover, Poly pretreatment down-regulated the expression of extracellular matrix (ECM) protein fibronectin (FN), profibrotic factor VEGF, proinflammatory cytokines MCP-1 and Rantes in the obstructed kidney. Similarly, the incubation of SV40-MES13 cells with Poly significantly inhibited Ang II-induced elevation in accumulation and expression level of FN and attenuated Ang II-evoked up-regulation in protein expression of MCP-1 and Rantes.

Conclusions: Our study isolated and identified a naturally occurring heteropolysaccharide in FLL and revealed its potential in protecting the kidneys from fibrosis.

Keywords: Angiotensin II, Fibrosis, *Fructus Ligustri Lucidi*, Kidney, Polysaccharide

Abbreviation

Ang II, angiotensin II; ARB, angiotensin type I receptor blocker; Ara, arabinose; CKD, chronic kidney disease; COSY, correlated spectroscopy; ECL, enhanced chemiluminescence; ECM, extracellular matrix; FLL, *Fructus Ligustri Lucidi*; FN, fibronectin; Gal, galactose; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GC-MS, gas chromatography-mass spectrometry; HMBC, heteronuclear multiple-bond correlation; HSQC, heteronuclear single quantum coherence; LLP, *Ligustrum Lucidum* polysaccharide; MCP-1, monocyte chemotactic protein-1; Mw, weight-average molecular weight; NMR, nuclear magnetic resonance; PAS, Periodic Acid-Schiff; PLLP, purified *Ligustrum Lucidum* polysaccharide; PMAA, partially methylated alditol acetate; Poly-H, high dose of FLL heteropolysaccharide; Poly-L, low dose of FLL heteropolysaccharide; RAS, renin-angiotensin system; UUO, unilateral ureteral obstruction; UV, ultraviolet; VEGF, vascular endothelial growth factor.

1. Introduction

Tubulointerstitial fibrosis is a chronic and progressive process affecting renal function due to aging (Denic et al., 2016) and/or chronic kidney disease (CKD) (Humphreys, 2018) independent of the underlying etiology (Nastase et al., 2018). A number of recent preclinical advances have clarified the cellular and molecular mechanisms underlying renal fibrosis, such as the signaling of angiotensin II (Ang II), which is the multiple-active peptide in the renin-angiotensin system (RAS). We have ever elucidated that treatment of primary renal tubular cells with Ang II would stimulate the production of profibrogenic factors (Zhang et al., 2010), and that the activity of tissue RAS was induced in the kidney of mice with hyperglycemia-induced nephropathy (Zhang et al., 2009; Zhang et al., 2008). Currently there are not yet any targeted therapies that could slow down (Humphreys, 2018) or reverse (Nastase et al., 2018) the development of renal fibrosis.

The significance of natural products as a source of potential drugs has been well recognized (Mahdi et al., 2022; Yousefi et al., 2021). Accumulating evidences have demonstrated the beneficial effects of natural products and functional ingredients on kidney fibrosis (Li et al., 2021; Ren et al., 2016). *Fructus Ligustri Lucidi* (FLL), the fruit of *Ligustrum lucidum* Ait. (Oleaceae) which is recorded as a tonic and dietary supplement that possesses the homology of medicine and food (He et al., 2018), has been traditionally used mainly to treat ailments such as menopausal problems, blurred vision, tinnitus, rheumatic pains, palpitations, backache, insomnia as well as to alleviate age-related symptoms (Zhang et al., 2006). It is also a commonly prescribed

component in traditional kidney-tonifying herbal formula and even appears as a feed additive in livestock nutrition (Li et al., 2017). As one of the kidney-tonifying herbs and in accordance with the “kidney governing bones” principle of Chinese medicine, emerging pieces of evidence suggested the protective effects of FLL on bone disorders like osteoporosis (Che et al., 2016; Sha et al., 2017). Our study found that treatment of FLL could improve depressive-like behavior by suppressing neuroinflammation *via* acting on tissue RAS in mice (Feng et al., 2020). Thus, we sought to clarify the effects of FLL on Ang II-induced nephropathy.

The main chemical constituents of FLL include flavonoids, secoiridoids, and triterpenes, which are responsible for the pharmacological activities of FLL (Chen et al., 2013; He et al., 2018). Recently, increasing attention has been paid to polysaccharides as an important source of bioactive natural products. Numerous researches have indicated the bioactivities of natural polysaccharides and promoted the application of polysaccharides in the prevention and treatment of diseases (Yu et al., 2018). One literature has ever reported extraction, isolation, and coagulative activity of polysaccharides from *Ligustrum lucidum* flowers (Yin et al., 2017). However, whether the natural polysaccharides exist in the fruit of *Ligustrum lucidum* and whether this component possesses an anti-fibrotic activity remain elusive.

Our purpose in this study was to explore the polysaccharide from FLL, clarify its chemical characteristic, and investigate its anti-fibrotic activity in kidney. A mice model of unilateral ureteral obstruction (UUO) was used to evaluate the nephroprotective effects of FLL polysaccharide since Ang II signaling is one of the

most crucial pathological mechanisms for renal fibrosis of UUO mice (Zhang et al., 2010). Additionally, the mouse glomerulus mesangial cells (SV40-MES13) were also applied to investigate the action of FLL polysaccharide on Ang II-evoked fibrosis.

2. Materials and methods

2.1. Polysaccharide preparation

The crude *Ligustrum Lucidum* polysaccharide (LLP) was prepared according to our previous report (Wang et al., 2021). Briefly, the dried and powdered (80-mesh) FLL (Hangzhou Tongjuntang Medicine Material Co., Ltd, China) was refluxed twice in 95% (v/v) ethanol at a ratio of 1:10 (g/mL) for 2h to remove lipids. The degreased powder was extracted in boiling water at a ratio of 1:10 (g/mL) for 2h twice. The extracts were filtered, combined, and concentrated. The LLP was precipitated by three volumes of 95% (v/v) ethanol and deproteinized by Sevag reagent (chloroform/butanol, v/v=4:1). After lyophilization, the samples (800 mg) were separated by a DEAE-cellulose DE52 (2.6 cm × 40 cm) column (GE Healthcare, Boston, MA, USA), and eluted stepwise with distilled water, 0.2 mol/L, 0.5 mol/L, and 1.0 mol/L NaCl aqueous solution, at a flow rate of 1.0 mL/min, leading to a collection of four fractions LLP-1, LLP-2, LLP-3, and LLP-4, respectively. The LLP-1 fraction was dialyzed against water (the molecular weight (M_w) cutting off of 3500 Da) for 48h and isolated by Sephacryl S-200 HR column (1.6 × 80 cm, GE Healthcare) with water at a flow rate 1.0 mL/min. The purified *Ligustrum Lucidum* polysaccharide (PLLP-1) was finally obtained. The phenol-sulfuric method was applied to determine the carbohydrate content. The regression equation was as follows: A_{490nm} = 0.9076*x* + 0.0895 with a correlation coefficient of 0.9915 (*x*: the carbohydrate content; A_{490nm}: the absorbance at 490 nm).

2.2. Purity analysis for PLLP-1

The purity of PLLP-1 was determined by ultraviolet (UV) absorption spectrophotometry (Vanavil et al., 2020). Briefly, the PLLP-1 aqueous solution (1.0 mg/mL) was analyzed by a UV-2600 UV-vis spectrophotometer (Shimadzu Co. Ltd., Kyoto, Japan). The wavelength range was set as 190-400 nm.

2.3. Molecular weight analysis

M_w of PLLP-1 was determined by gel-permeation chromatography (Lv et al., 2021) on an LC20 system (Shimadzu Co. Ltd.) equipped with an Alltech evaporative light scattering detector (Grace Alltech Co. Ltd., Chicago, IL, USA) using a TSK-Gel 3000 PWxl separation column (300 × 7.8 mm, 7 μm, Tosoh Co. Ltd., Kyoto, Japan). The mobile phase was water at a flow rate of 0.8 mL/min. The standard curve was established by a series of dextran standards (Merck KGaA, Darmstadt, Germany) with known molecular weights (M_w = 1.3 KDa, 5.2 KDa, 23.8 KDa, 147.6 KDa, 409.8 KDa, and 667.8 KDa, respectively). The calibration equation was as follows: log M_w = -0.5583x + 9.1424 (x: the retention time, correlation coefficient: 0.9976).

2.4. Monosaccharide composition analysis

The composition of monosaccharides in PLLP-1 was assessed by ion chromatography (Liu et al., 2020). Briefly, the PLLP-1 sample (10 mg) was hydrolyzed with trifluoroacetic acid (3 mol/L) at 120°C for 3 h. The dried residue was dissolved in water (5 mL) and underwent centrifugation (12000 rpm, 5 min), the

supernatant was analyzed by an ICS 5000 system equipped with an electrochemical detector (ThermoFisher Co. Ltd., New York, USA) and a Carbopac PA20 column (150 × 3 mm, 6 μm, ThermoFisher Co. Ltd.). Other detection conditions included the sample volume (5 μL), the column temperature (30°C), and the mobile phase (15 mmol/L NaOH and 100 mmol/L sodium acetate).

2.5. Gas chromatography-mass spectrometry analysis

The methylation of PLLP-1 was performed as described in Li et al. (2021). Briefly, the PLLP-1 sample (3.0 mg/mL in dimethyl sulfoxide) was incubated with methyl iodide and sodium hydroxide for 1h. The methylated polysaccharide was hydrolyzed by trifluoroacetic acid (2 mol/L, 1 mL) for 1.5h and dried through rotary evaporation. The residue was reduced by sodium borohydride, neutralized with acetic acid, and dried at 100°C. Subsequently, the dried residue was mixed with acetic anhydride (1 mL) at 100°C for 1h and analyzed by a QP 2010 gas chromatography-mass spectrometry (GC-MS, Shimadzu Co. Ltd.) using an RXI-5 SIL MS capillary separation column (30 m × 0.25 mm, 0.25 μm, Shimadzu Co. Ltd.). The temperature program was as follows: initial column temperature was 120°C, followed by a programmed increase from 120°C to 250°C at 3°C/min, and held for 5 min.

2.6. Nuclear magnetic resonance analysis

Nuclear magnetic resonance (NMR) analysis was performed as described in Li et al. (2021). Briefly, the PLLP-1 sample was dissolved in D_2O (100 mg/mL) and analyzed

by an Avance 600-MHz NMR spectrometer (NMR, Bruker Co. Ltd., Rheinstetten, Germany). One-dimensional NMR spectra (^{1}H, ^{13}C, and Dept135) and two-dimensional NMR spectra [correlated spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple-bond correlation (HMBC)] were adopted to determine the structural features of PLLP-1. Data were analyzed by MestRe Nova 6.1.0 software (Mestrelab Research S.L., Santiago de Compostela, Spain).

2.7. Animal treatments

Ten-week-old male C57BL/6J mice (23-25 g) were randomly assigned to three groups including the Sham group (n = 8), and the UUO group treated with vehicle (n = 8) and heteropolysaccharide (Poly, 100 mg/kg, n = 8) of FLL. Briefly, UUO was performed under isoflurane anesthesia in which a midline incision was made and the left ureter was exposed and tied off at two points. Sham operation was performed similarly but without ureter ligation. For the Poly treatment group, mice underwent daily intraperitoneal injection with FLL heteropolysaccharide starting from 7 days before UUO surgery until day 7 after surgery. All mice were killed on day 7 after the surgical operation by cardiac exsanguination. Kidneys were collected for analysis. All animal procedures were performed in accordance with the NIH Guide for Care and Use of Laboratory Animals. The animal study protocol was reviewed and approved by the Animal Ethics Committee of Longhua Hospital, affiliated with Shanghai University of Traditional Chinese Medicine.

2.8. Wet weight index of kidney

The wet weight of the obstructed kidney was recorded. The kidney index was calculated by dividing the weight of the obstructed kidney by the bodyweight of the mice.

2.9. Histopathological staining

The separated kidneys were fixed in 4% formaldehyde/PBS (pH 7.2) overnight and embedded in paraffin subsequently. Kidney sections were cut at 4 μm, and the sections were stained with Periodic Acid-Schiff (PAS, Solarbio, Beijing, China) and Masson's trichrome (Solarbio), respectively. PAS staining was used to score glomerular fibrosis on a scale of 0-4 as described in previous study (Zhang et al., 2008). The area in renal interstitial fibrosis was also counted from images stained by Masson's trichrome.

2.10. Cell culture and treatment

The mycoplasma-free murine mesangial cells SV40-MES13 were purchased from National Collection of Authenticated Cell Cultures (NCACC, Shanghai, China). The cells were cultured with 10% fetal bovine serum (Biosera, Nuaillé, France) and 1% penicillin/streptomycin (Biosera) at 37°C in a humidified atmosphere with 5% CO_2. The cells were seeded in a 24-well plate to make the cell slides, followed by immunofluorescence staining, or in a 6-well plate for measuring protein expression.

The SV40-MES13 cells were treated with vehicle, Ang II (10^{-7} M) in the presence or absence of angiotensin type I receptor blocker (ARB) telmisartan (10^{-6} M) or with a low dose (50 μg/mL, Poly-L) and high dose (200 μg/mL, Poly-H) of FLL heteropolysaccharide. The cells were pretreated with Poly-L, Poly-H, and ARB for 24h before the induction of Ang II for 48h, followed by harvesting for bioassays.

2.11. Immunofluorescence analysis

The SV40-MES13 cell slides were fixed with 4% formaldehyde in PBS for 15 min at room temperature, blocked, and incubated with rabbit anti-fibronectin (FN) antibody (1:50, Abcam, Cambridge, UK), followed by the incubation with both Alexa Fluor 488-conjugated goat polyclonal anti-rabbit IgG (Abcam) and phalloidin (Cell Signaling Technology, Boston, MA, USA). The images in the stained slides were observed and captured under a microscope VS-120 (Olympus, Japan).

2.12. Western blotting

Proteins from mice renal tissues and SV40-MES13 cells were extracted by RIPA lysis buffer containing protease inhibitor cocktail (Beyotime). The protein concentration of all lysates was determined using a BCA assay (Beyotime). After electrophoresis, the proteins were transferred onto PVDF membranes (Millipore, Darmstadt, Germany), blocked with 5% BSA in TBST for 1h at room temperature. The membranes were incubated at 4°C overnight with primary antibodies, including rabbit anti-FN (1:1000, Abcam), rabbit anti-MCP-1 (1:1000, Abcam), and rabbit

anti-Rantes (1:1000, Abcam). After 3 washes with TBST, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:3000, Beyotime), and developed using enhanced chemiluminescence (ECL) solution (Millipore). The bands in blots were quantified by measuring the intensity of the signals using Image-Pro Plus (version 6.0) and normalized to the β-actin signal detected by mouse monoclonal antibody (Sigma-Aldrich, St Louis, MO, USA).

2.13. RT-PCR

Total RNA was isolated using TRIzol reagents (Thermo Fisher Scientific, Waltham, MA, USA). After synthesizing cDNA by reverse transcription with 2 μg of RNA using MMLV reverse transcriptase (Thermo Fisher Scientific), the cDNA was used as a template for PCR amplification using a DNA Engine (ABI). The program was set up as follows: an initial step at 95°C for 2 min, 40 cycles of 95°C for 15 sec, 56°C for 25 sec and 72°C for 20 sec. The primer sequences used in this study were as follows: FN, forward: cgaggtgacagagaccacaa, reverse: ctggagtcaagccagacaca; monocyte chemotactic protein-1 (MCP-1), forward: gctcagccagatgcagttaa; reverse: tcttgagcttggtgacaaaaact; vascular endothelial growth factor (VEGF), forward: gagcagaagtcccatgaagtgat, reverse: atccgcatgatctgcatggt; and Rantes, forward: ccctcaccatcatcctcact, reverse: ccacttcttctctgggttgg. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control was used to normalize the data to analyze the relative expression of the target genes.

2.14. Statistical analysis

Data are expressed as means ± SEM. Statistical analyses using one-way ANOVA followed by Newman-Keuls multiple comparison tests were performed by GraphPad Prism 8.0. *P* value <0.05 was considered statistically significant.

3. Results

3.1. Physicochemical compositions of PLLP-1

The preparation procedure diagram was shown in Fig. 1A. The extraction yield of LLP was 12.52% relative to the dried material. After isolation on the DEAE-cellulose DE52 column, the LLP was separated into four fractions, LLP-1, LLP-2, LLP-3, and LLP-4 (Fig. 1B). The yields of LLP-1, LLP-2, LLP-3, and LLP-4 were respectively calculated to be 1.23%, 0.21%, 0.28%, and 0.15% of the dried material. LLP-1, the most abundant component of LLP, was further purified with the gel-filtration column to obtain the pure polysaccharide PLLP-1 (Fig. 1C).

3.2. Molecular weight and methylation of PLLP-1

The carbohydrate content of PLLP-1 was 97.45% and there was a single symmetrical peak at 6.81 min (Fig. 1D). Its M_w was 15.0 KDa. There was no UV absorption at 260 or 280 nm, indicating neither proteins nor nucleic acids were found in PLLP-1 (Fig. 1E).

PLLP-1 was mainly composed of arabinose (Ara) and galactose (Gal) in a molar ratio of 0.73:0.27. The methylated PLLP-1 was measured by GC-MS to determine the types of sugar residues (Table 1). Seven peaks in the GC chromatogram were observed with retention times at 16.0, 17.4, 19.7, 21.2, 25.1, 32.0, and 38.1 min, indicating T-Ara*f*, T-Ara*p*, 1,3-Ara*f*, 1,5-Ara*f*, 1,3,5-Ara*f*, 1,6-Gal*p*, and 1,3,6-Gal*p*, respectively, by comparing the mass spectrometry (MS) fragment ions of each peak

(Fig. S1) with the Complex Carbohydrate Research Center (CCRC) spectral database in Partially Methylated Alditol Acetate (PMAA) (Ciucanu, 2006) and the previous literature (Wu et al., 2020).

3.3. NMR analysis

We used both 1D and 2D NMR spectra to determine the structure of PLLP-1. The ^{1}H NMR (Fig. S2A) and ^{13}C NMR (Fig. S2B) spectra of PLLP-1 demonstrated that most proton and carbon signals of this arabinan ranged from δ3.50-5.20 ppm and δ60-110 ppm, respectively. In the ^{13}C NMR spectrum, the anomeric carbon signals were evident from δ99.8 to δ109.2 ppm, while the HSQC spectrum (Fig. S2C) indicated that the anomeric carbon signals at δ109.2, 107.4, 107.1, 107.0, 103.1, 99.8 and 99.0 ppm were associated with anomeric proton signals at δ5.20, 5.03, 5.10, 5.13, 4.48, 4.79, 5.06 ppm, respectively. Based on HSQC and the data reported in the literature, the chemical shift signals were assigned to residue A (T-α-Ara*f*) (Deng et al., 2020) at δ5.20 and 109.2 ppm, residue B (1,3,5-α-Ara*f*) (Chen et al., 2021) at δ5.13 and 107.0 ppm, residue C (1,3-α-Ara*f*) (Huo et al., 2020) at δ5.10 and 107.1 ppm, residue D (T-α-Ara*p*) (Li et al., 2020) at δ5.06 and 99.0 ppm, residue E (1,5-α-Ara*f*) (Chen et al., 2021) at δ5.03 and 107.4 ppm, residue F (1,6-α-Gal*p*) (Zhou et al., 2021) at δ4.79 and 99.8 ppm, and residue G (1,3,6-β-Gal*p*) (Wang et al., 2021) at δ4.48 and 103.1 ppm. These results were consistent with those from the GC-MS analysis. Then, the COSY spectra (Fig. S2D) were used to analyze the information of other proton signals in PLLP-1. For example, residue A had an anomeric signal (H1) at δ5.20 ppm,

with H2 of this residue having a ^{1}H resonance indexed to δ4.17 ppm according to the COSY spectrum. The signals of C2-C5 of Ara and C2-C6 of Gal at δ61.1-84.0 ppm and δ69.7-75.8 ppm were determined by HSQC and Dept135 (Fig. S2E). Specifically, H5/C5 (δ3.73/61.1 ppm), H5/C5 (δ3.59/69.6 ppm), H5/C5 (δ3.66/62.8 ppm), H5/C5 (δ3.85/66.3 ppm) and H5/C5 (δ3.59/66.8 ppm) could be assigned to T-α-Ara*f*, 1,3,5-α-Ara*f*, 1,3-α-Ara*f*, T-α-Ara*p*, and 1,5-α-Ara*f*, respectively. H6/C6 (δ3.64/71.7 ppm) and H6/C6 (δ 4.08/69.7 ppm) could be assigned to 1,6-α-Gal*p* and 1,3,6-β-Gal*p*. The chemical shifts of the protons and corresponding carbon atoms were summarized in Table 2.

HMBC detection (Fig. S2F) was conducted to analyze the glycosidic linkage of PLLP-1. The cross-peaks at D(H1)/G(C3) (δ5.06/75.8 ppm) indicated a connection between C1 of T-α-Ara*p* and C3 of 1,3,6-β-Gal*p*. In addition, we detected inter-residual cross-peaks, F(H1)/B(C5) (δ4.79/69.6 ppm), G(H6)/B(C1) (δ4.08/107.0 ppm), G(H1)/G(C6) (δ4.48/69.7 ppm), E(H5)/G(C1) (δ3.59/103.1 ppm), E(H1)/E(C5) (δ5.03/66.8 ppm), D(H1)/C(C3) (δ5.06/79.0 ppm), E(H1)/F(C6) (δ5.03/71.7 ppm), A(H1)/C(C3) (δ5.20/79.0 ppm), and C(H1)/B(C3) (δ5.10/84.0 ppm), indicating that C1 of F (1,6-α-Gal*p*) was connected to the 5-position of B (1,3,5-α-Ara*f*), C6 of G (1,3,6-β-Gal*p*) was connected to 1-postion of B (1,3,5-α-Ara*f*), C1 of G (1,3,6-β-Gal*p*) was connected to the 6-position of G (1,3,6-β-Gal*p*), C5 of E (1,5-α-Ara*f*) was connected to the 1-position of G (1,3,6-β-Gal*p*), C1 of E (1,5-α-Ara*f*) was connected to 5-position of E (1,5-α-Ara*f*), C1 of D (T-α-Ara*p*) was connected to 3-postion of C (1,3,6-β-Gal*p*), C1 of E (1,5-α-Ara*f*) was connected to 6-position of F (1,6-α-Gal*p*),

C1 of A (T-α-Ara*f*) was connected to 3-postion of C (1,3-α-Ara*f*), and C1 of C (1,3-α-Ara*f*) was connected to 3-postion of B (1,3,5-α-Ara*f*). Based on these chemical and spectroscopic analyses, the proposed PLLP-1 structure was shown in Fig. 2. The main linkages of PLLP-1 contained →)1-α-Ara*f*(5→, →)1-α-Gal*p*(6→ and →)1-β-Gal*p*(6→ residues and the side chains were α-Ara*f*(1→ or α-Ara*p*(1→ linked at the C3-position of →)1-β-Gal*p*(6→ and α-Ara*p*(1→3)-α-Ara*f*(1→ linked at the C3-position of→)1-α-Ara*f*(5→.

3.4. The heteropolysaccharide improved fibrotic phenotype in the obstructed kidney

The wet weight of the obstructed kidneys in UUO mice was significantly (Fig. 3A&D, $P < 0.001$) increased compared with that of Sham mice, whereas, the wet weight was not statistically different between heteropolysaccharide-treated UUO mice and vehicle-treated UUO mice.

Fibrosis in kidney was respectively evaluated by glomerular sclerosis using PAS staining (Fig. 3B) and tubulointerstitial fibrosis using Masson's trichrome staining (Fig. 3C). The semiquantitative scoring (on a scale of 0 to 4) for PAS staining confirmed that the UUO mice showed severe glomerular fibrosis (Fig. 3E, $P < 0.001$), while, the pretreatment of Poly dramatically ($P < 0.001$) abrogated glomerular fibrosis of the obstructed kidneys after 7 days of UUO.

Masson's trichrome staining was applied to dye collagen fibers with blue in the paraffin renal sections in order to characterize the degree of interstitial fibrosis. By quantifying the positive area of fibrosis, it was found that the lateral kidney after

UUO surgery showed severe tubulointerstitial fibrosis compared to the Sham mice (Fig. 3F, $P < 0.001$), and the pretreatment with Poly markedly ($P < 0.001$) reduced the intrarenal fibrotic area.

3.5. The heteropolysaccharide regulated the expressions of profibrotic and proinflammatory cytokines in the obstructed kidney

The molecular expressions (Fig. 4A&C) of profibrotic (VEGF) and proinflammatory (MCP-1 & Rantes) cytokines as well as of the extracellular matrix (ECM) protein (FN) were detected in the obstructed kidneys of UUO mice to examine the underlying mechanism involved in the reno-protective effects of Poly. As expected, we found that the transcription levels (Fig. 4B) of FN ($P < 0.05$), VEGF ($P < 0.05$), MCP-1 ($P < 0.001$), and Rantes ($P < 0.001$) were up-regulated in the UUO group, whereas the Poly pretreatment reversed the changes ($P < 0.01$) of these factors except for VEGF. In addition, the protein expressions of FN, MCP-1, and Rantes showed a pronounced rise (Fig. 4D, $P < 0.01$) in the UUO group in a comparison with those in the Sham group, and in accordance with the regulation of mRNA expression, the administration with Poly repressed ($P < 0.05$) the effects of UUO on these cytokines in the obstructed kidney.

3.6. The heteropolysaccharide reduced the abundance and expression of FN in murine mesangial cells

The production of ECM is a hallmark of the progression of organ fibrosis. Ang II is

recognized as a vital stimulator of the expression of profibrotic cytokines, especially since it could be synthesized in mesangial cells and lead to renal injury. Therefore, the abundance (Fig. 5A) and the expression (Fig. 6A) of FN were examined by immunofluorescence and immunoblotting, respectively, in murine mesangial cells SV40-MES13. Ang II produced an acute accumulation of FN (Fig. 5B, $P < 0.001$) and enhanced protein expression of FN (Fig. 6B, $P < 0.01$). The angiotensin type 1 receptor blocker (ARB) effectively blocked ($P < 0.01$) the induction of Ang II on FN, moreover, the pretreatments with both Poly-L and Poly-H could attenuate ($P < 0.001$) the fluorescence intensity of FN, and consistently, result in a drop ($P < 0.05$) of FN protein expression in Ang II-treated mesangial cells.

Besides the modulation of ECM fibrotic protein FN, the heteropolysaccharide Poly significantly abrogated Ang II-induced elevation of the expression of MCP-1 ($P < 0.001$) and Rantes ($P < 0.01$).

4. Discussion

Organ fibrosis is characterized by a progressive accumulation of fibrous tissue and by reduced remodeling that can lead to the impairment in the function of the affected organ (Albeiroti et al., 2015; Masola et al., 2020). This pathological process is quite common in several parenchymal organs such as the kidney, liver, and lungs (Masola et al., 2020). At present, organ fibrosis represents a real health emergency in the developed western countries and is a major challenge to global health, since a real anti-fibrotic therapy is not yet available in most cases (Masola et al., 2020; Roehlen et al., 2020). Hence, there is a huge unmet medical need for anti-fibrotic therapies to prevent the progression of organ fibrosis (Roehlen et al., 2020). Experimental research studies have shown that polysaccharides from natural sources displayed remarkable anti-fibrotic activities (Li et al., 2019). Thus, this study was performed to identify the polysaccharide contained in FLL and explore its protective effect against renal fibrosis.

In this study, high-performance gel permeation chromatography was utilized to assess the molecular weight of the purified polysaccharide, and ion chromatography analysis showed that its monosaccharide composition was Ara and Gal. In view of the compositions of polysaccharides in *Ligustrum lucidum*, one study found heteropolysaccharides and Gal-constituted homopolysaccharides isolated from its flowers (Yin et al., 2017), and the capillary zone electrophoresis with amperometric detection was applied to determine the composition of FLL polysaccharide (Wang et al., 2003). The present study further identified arabinogalactan from FLL

polysaccharide components and proposed the predicted chemical backbone structure. Previous functional studies have demonstrated that FLL polysaccharides could protect against lipopolysaccharide-induced inflammatory injury of Sertoli cells in rats (Yu et al., 2018) and improve immune functions of mice with hydrocortisone-induced immunosuppression (Shi et al., 2016). Our subsequent study was conducted to determine the anti-fibrotic effects of the identified heteropolysaccharide (Poly) in the kidney by *in vivo* and *in vitro* experiments.

The pretreatment of UUO mice with Poly markedly improved the renal fibrotic phenotype characterized by the decrease in scores for glomerular sclerosis and in the area of interstitial fibrosis. Furthermore, molecular detections indicated that Poly down-regulated the expression of profibrotic factor VEGF and reduced the production of FN, one of the major ECM proteins, as well as inhibited the expression of proinflammatory cytokines MCP-1 and Rantes, in the kidney of UUO mice, suggesting the suppressive effects of Poly on fibrosis and inflammation in UUO-induced kidney injury. So far, only naturally-occurring heteropolysaccharides composed of Ara and other monosaccharides have been reported to be able to repress renal fibrosis (Lian et al., 2021; Wu, 2019) and hepatic fibrosis (Ke et al., 2020). Our chemical and biological studies clarified the existence of heteropolysaccharides composed of Ara and Gal in FLL and revealed their potential in attenuating renal fibrosis in mice.

Ang II, an active peptide in RAS, could exert multiple actions in tissues. It is well elucidated that Ang II is one of the key profibrotic cytokines and could promote tissue

fibrosis *via* a signaling pathway for ECM accumulation (Zhang et al., 2010). Given the amelioration of FLL water fraction on the rise in expression of renin (a rate-limiting enzyme in RAS) and Ang II in the hypothalamus of mice after challenge with LPS injection (Feng et al., 2020), the *in vitro* effects of FLL heteropolysaccharide on SV40-MES13 with exposure to Ang II stimulation were studied. As a type of mouse glomerulus mesangial cells (Wu et al., 2021), SV40-MES13 is commonly applied for preclinical research on renal fibrosis, even the involvement of renin receptor in mesangial fibrosis was elucidated in this cell line (Hu et al., 2020; Narumi et al., 2018). In accordance with our previous study showing Ang II-induced epithelial-to-mesenchymal transition (EMT) in primary tubular cells (Zhang et al., 2010), the present immunofluorescence analysis displayed the accumulation of FN, a hallmark of fibrosis, in SV40-MES13 cells in response to Ang II treatment. Intriguingly, the incubation with the heteropolysaccharide Poly profoundly decreased Ang II-triggered production of FN and diminished Ang II-evoked up-regulation of MCP-1 and Rantes expressions, implying that the heteropolysaccharide of FLL could effectively relieve Ang II-initiated fibrosis and even inflammation in the kidney. However, the regulation of Poly on Ang II signaling still needs to be further dissected.

5. Conclusions

In conclusion, our present study discovered a heteropolysaccharide consisting of Ara and Gal from the fruit of *Ligustrum lucidum*, and the pharmacological studies

demonstrated its protective effect on renal fibrosis caused by UUO through interfering with Ang II signaling pathway. The potential application of FLL heteropolysaccharides and their clinical therapeutic efficacy as novel drugs or natural supplements for the management of renal fibrosis associated with CKD and in the treatment of fibrosis in other organs are worth further investigation.

CRediT authorship contribution statement

Jia-li Zhang: Performed the study and analyzed the results**. Chen Du:** Performed the study and data interpretation**. Christina Chui-Wa Poon:** Data interpretation and manuscript revision. **Ming-Chao He:** Performed the study and analyzed the results**. Man-Sau Wong:** Conceptualization and data interpretation**. Na-Ni Wang:** Performed the study, analyzed the results, and wrote the manuscript**. Yan Zhang:** Designed the study, contributed to the concept generation, and wrote and revised the manuscript**.** All authors approved the final version of the manuscript.

Declaration of competing interest

All authors state that they have no conflicts of interest.

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Figure legends

Figure 1. Preparation and analysis of PLLP-1. (A) The procedure of preparation and purification. (B) Elution profile on DEAE-cellulose DE52 column. (C) Elution profile on Sephacryl S-200 HR column. (D) Gel-permeation chromatogram. (E) UV spectrum of PLLP-1.

Figure 2. Chemical structure of PLLP-1.

Figure 3. The wet weight of obstructed kidneys and the phenotype of renal fibrosis. The UUO mice were intraperitoneally injected with FLL heteropolysaccharide (Poly, 100 mg/kg) 7 days prior to surgery and lasted for 7 days after the operation. (A) The unilateral kidneys were freshly removed, weighed, and photographed. The kidney index (D) was calculated based on the ratio of wet weight and body weight. PAS staining was performed to observe glomerular sclerosis (B, magnification, ×200) and the semiquantitative score (on a scale of 0 to 4) was presented (E). Masson's trichrome staining was conducted to indicate tubulointerstitial fibrosis (C, magnification, ×200) and the fibrotic area with positive staining in blue was expressed as a percentage (F). Values were expressed as means ± SEM (n = 8). *** $P < 0.001$, *vs*. Sham; ### $P < 0.001$, *vs*. UUO.

Figure 4. Transcriptional level of profibrotic and proinflammatory cytokines. A,

RT-PCR detection. B, the quantitative data on expression of target mRNA. C, immunoblotting. D, the quantitative data on expression of target protein. Values were expressed as means ± SEM (n = 6). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, *vs*. Sham; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, *vs*. UUO.

Figure 5. Immunofluorescence detection on FN. The SV40-MES13 cells were treated with ARB telmisartan (10^{-6} M), low dose (50 μg/mL, Poly-L) and high dose (200 μg/mL, Poly-H) of FLL heteropolysaccharide with the induction of Ang II (10^{-7} M). A, Immunofluorescence staining on FN. B, the quantification on the area of the fluorescent signal. *** $P < 0.001$, *vs*. Vehicle; ### $P < 0.001$, *vs*. Ang II.

Figure 6. Protein expression of FN, MCP-1 and Rantes. The SV40-MES13 cells were treated with ARB telmisartan (10^{-6} M), low dose (50 μg/mL, Poly-L) and high dose (200 μg/mL, Poly-H) of FLL heteropolysaccharide with the induction of Ang II (10^{-7} M). A, immunoblotting. B, the quantitative data on expression of target protein. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, *vs*. Vehicle; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, *vs*. Ang II.

Figure legends (Supplement)

Fig. S1. Mass spectra of PMMA for Residue A-G.

Fig. S2. NMR spectra of PLLP-1. (A) ^{1}H NMR. (B) ^{13}C NMR. (C) HSQC (D) COSY (E) Dept135. (F) Superimposition of HSQC (gray) and HMBC (red/yellow) spectra.

Table 1 Methylation analysis for PLLP-1 by GC-MS

RT (min)	Methylated sugar	Mass fragments (m/z)	Type of linkage	Molar ratio
16.0	1,4-Di-O-acetyl-2,3,5-tri-O-methyl- arabinitol	43,71,87,101,117,129,145,161	Terminal-Ara*f*	17.8%
17.4	1,5-Di-O-acetyl-2,3,4-tri-O-methyl arabinitol	43,71,87,101,117,129,145,161	Terminal-Ara*p*	8.8%
19.7	1,3,4-Tri-O-acetyl-2,5-di-O-methyl arabinitol	43,87,99,101,117,201,233	1,3-Ara*f*	4.6%
21.2	1,4,5-Tri-O-acetyl-2,3-di-O-methyl arabinitol	43,71,87,99,117,189,233	1,5-Ara*f*	36.2%
25.1	1,3,4,5-Tretra-O-acetyl-2-O-methyl arabinitol	43,58,85,99,117,217,261	1,3,5-Ara*f*	5.3%
32.0	1,5,6-Tri-O-acetyl-2,3,4-trio-O-methyl galactitol	43,71,87,101,117,129,161,173,189, 233	1,6-Gal*p*	4.6%
38.1	1,3,5,6-Tretra-O-acetyl-2,4-di-O-methyl galactitol	43,87,117,129,189,173,233,305	1,3,6-Gal*p*	22.6%

Table 2 ^{1}H NMR and ^{13}C NMR chemical shifts of the sugar residues of PLLP-1

	H1/C1	H2/C2	H3/C3	H4/C4	H5/C5	H6/C6
T-α-Ara*f*	5.2	4.17	3.85	4.08	3.73	
(A)	109.2	81.3	76.7	81.2	61.1	
1,3,5-α-Ara*f*	5.13	4.16	4.03	4.04	3.59	
(B)	107	80.8	84	82.3	69.6	
1,3-α-Ara*f*	5.1	4.07	4.08	4.25	3.66	
(C)	107.1	80.1	79	79.4	62.8	
T-α-Ara*p*	5.06	4.23	4.04	3.94	3.85	
(D)	99	68.7	69.3	69	66.3	
1,5-α-Ara*f*	5.03	4.08	3.94	4.09	3.59	
(E)	107.4	83.8	76.5	82.9	66.8	
1,6-α-Gal*p*	4.79	3.92	3.9	3.7	3.83	3.64
(F)	99.8	69.8	70.2	75.8	73.6	71.7
1,3,6-β-Gal*p*	4.48	3.62	3.67	4.05	3.78	4.08
(G)	103.1	71.2	75.8	69.9	73.7	69.7