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Structure and biological activities of a pectic polysaccharide from *Mosla chinensis* Maxim. cv. Jiangxiangru

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ABSTRACT

A water-soluble pectic polysaccharide (MP-A40) was isolated and purified from *Mosla chinensis* Maxim. cv. Jiangxiangru for the first time, with a molecular weight of 32,600 Da. MP-A40 was comprised of 68.63% galacturonic acid and 13.05% neutral sugar. In addition, arabinose, galactose, rhamnose, mannose and glucose composed the neutral sugar in a relative ratio of 4.94, 3.07, 2.13, 1.62 and 1.29% of the dry weight of MP-A40, respectively. Structural characterization of MP-A40 was investigated by methylation analysis and 1D/2D NMR spectroscopy. From the results, the structure of MP-A40 was revealed as follows: 1,4-linked α-D-Gal*p*A and 1,4-linked α-D-Gal*p*A6Me interspersed with rare t-Ara*f* (0.60%), t-Rha*p* (1.67%) and t-Gal*p*A (10.15%). Esterification assay showed that about 32% of the carboxylic groups in GalA residues existed as methyl ester. In addition, MP-A40 could inhibit the growth of human leukemic cell line K562 and stimulate nitric oxide production from RAW 264.7 macrophages both in dose-dependent manners.

1. Introduction

Mosla chinensis Maxim. cv. Jiangxiangru, as a member of *Labiatae* family, commonly known as *Elsholtzia ciliata (Thunb) Hyland*, is a well-known traditional medicinal vegetable in southern China with an early historical record in "MING YI BIE LU". It is also distributed in other countries such as Vietnam, India and Japan. In addition to be widely used as eatable vegetable, it is also known as a popular folk medicine for the treatment of many diseases, such as cold, fever, diarrhea, dysentery, digestion disorder, vomiting, stroke and edema (China Pharmacopoeia Committee, 2005). In recent years, *M. chinensis* Maxim. cv. Jiangxiangru has attracted great attention due to the bioactivities itself as well as its components, including essential oil (Li J.E. et al., 2010), polysaccharides (Li J.E., Nie, Yang, Qiu, & Xie, 2011), inorganic elements (Huang et al., 2009) and flavonoids (Hu, Xie, Zhang, & Shu, 2010).

Polysaccharides are generally known as a kind of biomacromolecules (Cheung et al., 2009; Jung, Bae, Lee, & Lee, 2011; Tehila, Margalit, Dorit, Shlomo, & Shoshana, 2005), that are widely used in the food, pharmaceutical and medicine industries (Moradali, Mostafavi, Ghods, & Hedjaroude, 2007). Polysaccharides exerted their antitumor effect through activating the immune system of the host animal (Wu et al., 2012). Among polysaccharides, pectins are an important family of heterogeneous polysaccharides isolated from the plant cell wall (Albersheim, Darvill, O'Neill, Schols, & Voragen, 1996). The complex structures of pectins consist of homogalacturonan (HG) and rhamnogalacturonans (RG-I and RG-II) (Muzzarelli et al., 2012; Pilnik & Voragen, 1970). The aim of the present study was to investigate the structure of the repeating unit of a pectic polysaccharide (MP-A40) isolated from *M. chinensis* Maxim. cv. Jiangxiangru, and to explore its antitumor and immunomodulatory activities *in vitro*.

2. Materials and methods

2.1. Plant materials and chemicals

Whole plants of *M. chinensis* Maxim. cv. Jiangxiangru were purchased from Zhangshu, Jiangxi, China. Q Sepharose™ Fast Flow was purchased from Amersham Biosciences (Uppsala, Sweden). DP3, DP5, DP7, and a pullulan standard P-100 with molecular weights of 504 Da, 828 Da, 1152 Da and 1.07×10^5 Da respectively, were obtained from Sigma–Aldrich (Bellefonte, PA, USA). Pectin standards with different degrees of esterification (26, 59, 76.5 and 94%) were purchased from Sigma–Aldrich (St. Louis, MO, USA). All the reagents used in the study were of analytical grade.

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2.2. Isolation and purification of the polysaccharide MP-A40

M. chinensis Maxim. cv. Jiangxiangru whole plants were ground into powder and air dried before extraction. 100 g ground sample was soaked in 80% ethanol to remove small molecules such as pigments, lipophilic molecules and oligosaccharides (Chen, Cao, & Song, 1996). The polysaccharide was isolated according to our previous method (Li J.E. et al., 2011). Then polysaccharide was deproteinated by the Sevag method (Staub, 1965). After that, about 10.5 g crude polysaccharide (CMP) was obtained.

For purification, 400 mg CMP was loaded onto a pre-equilibrated Q Sepharose™ Fast Flow ion exchange column (XK 50/30 Column, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and eluted at a flow rate of 6 mL/min for 200 min using deionized water as mobile phase. The eluate (neutral polysaccharide, defined as MP-N) was collected from 100 to 170 min. The column was successively washed with 1.5 L 1 M NaCl solution using the same flow rate (6 mL/min), and a second eluate (an acidic fraction, defined as MP-A) was also collected. At last, 1 L 2 M NaCl solution was allowed to pass through the column in order to remove the proteins and pigments ionically bound to the column material (Qian, Cui, Wu, & Goff, 2012). MP-N and MP-A were concentrated by rotary vacuum evaporator at 55 °C, dialyzed (molecular weight cut-off 3.5 kDa, VWR Scientific, Spectrum Laboratories, Inc., Rahcho Dominguez, CA) against distilled water at room temperature for 72 h, and then freeze-dried. MP-A was further separated into four fractions designated as MP-A40, MP-A50, MP-A60, and MP-A80 by gradient precipitation with 40, 50, 60, and 80% of ethanol (v/v), respectively (Kang et al., 2011). Among them, MP-A40 exhibited a single symmetrical peak on the high performance size-exclusion chromatography (HPSEC) profile, indicating a high purity.

2.3. Physicochemical properties of MP-A40

2.3.1. Determination of neutral sugar, uronic acid, and protein

The content of neutral sugar of MP-A40 was determined by phenol-sulfuric acid method with slight modifications (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The uronic acid content was measured by m-hydroxydiphenyl colorimetric method (Blumenkrantz & Asboe-Hansen, 1973). Protein analysis was performed on an NA2100 Nitrogen and Protein Analyzer (Strada Rivoltana, Milan, Italy) according to the manual of this equipment (Qian et al., 2012). The protein content was calculated by multiplying a conversion factor of 6.25. All chemical analyses were carried out in triplicate.

2.3.2. Determination of molecular weight and its distribution

The average molecular weights (Mw) of MP-A40 was determined by high performance size-exclusion chromatography (HPSEC) coupled with multiple detectors: a differential pressure viscometer (DP), a refractive index detector (RI), a UV detector, a right angle laser light scattering detector (RALLS), and a low angle laser light scattering detector (LALLS). The system was equipped with three columns (AQ00192, AQ00191, AQ00194, PolyAnalytik Inc., London, Ontario, CA) in series connection. The columns, viscometer, and RI detector were maintained at 40 °C. The mobile phase was made of 0.1 M $NaNO_3$ solution containing 0.03% (w/w) NaN_3 with a flow rate of 0.6 mL/min. After being filtered through a 0.45 μm membrane, 100 μL sample was injected into the system.

Mw was estimated by a calibration curve prepared with three oligosaccharide standards (DP3, DP5 and DP7) and a pullulan standard (P-100). Data were collected and analyzed using the OminiSEC 4.6.1 software (Malvern company, Worcestershire, UK).

2.3.3. Determination of monosaccharide composition

The monosaccharide compositions of MP-A40 was analyzed on a high performance anion exchange chromatography system (HPAEC) (Dionex, DX500 Sunnyvale, CA, USA) coupled with a pulsed amperometric detector (HPAEC-PAD). 10 mg sample was stirring in 1 mL 12 M H_2SO_4 at room temperature for 30 min according to the method of Mopper et al. (1992) with some modifications. Then 5 mL distilled water was added, and the solution was heated at 100 °C for 2 h. Monosaccharide standard mixtures (L-rhamnose, L-arabinose, D-galactose, D-glucose, and D-mannose) were well prepared with concentrations of 30, 50, 80, 100 μg/mL, respectively. All the tests were performed three times.

2.3.4. Determination of the degree of esterification by FT-IR spectroscopic method

FTIR spectrum of MP-A40 was obtained using a Golden-gate Diamond single reflectance ATR in a FTS 7000 FT-IR spectrometer equipped with a DTGS detector (DIGILAB, Randolph, MA, USA). The spectrums were recorded at the absorbance mode from 4000 to 400 cm^{-1} (mid infrared region) at a resolution of 4 cm^{-1} with 128 co-added scans.

The degree of esterification (DE) was defined as the ratio of the area of the band at 1730 cm^{-1} (corresponding to the number of esterified carboxylic groups) over the sum of the areas of the bands at 1730 and 1600 cm^{-1} (corresponding to the number of total carboxylic groups). DE was calculated according to the method of Singthong, Cui, Ningsanond, and Goff (2004), using four pectin standards with different DE values of 26, 59, 76.5 and 94%, respectively.

2.4. Methylation analysis

Uronic acids were reduced to neutral sugar before methylation as described previously by York, Darvill, McNeil, Stevenson, and Albersheim (1986) with minor modifications. Methylation analysis was conducted according to the method of Cui W., Eskiin, Biliaderis, and Marat (1996).

The resultant partially methylated alditol acetates (PMAA) were injected into a gas chromatography-mass spectrometry (GC-MS) system (Agilent technology 6890N GC/5973 insert MSD) fitted with a SP-2330 (Supelco, Bellefonte, PA, USA) capillary column (30 m × 0.25 mm, 0.2 μm film thickness, 160–210 °C at 2 °C/min, and then 210–240 °C at 5 °C/min) and equipped with an ion trap MS detector.

2.5. NMR spectroscopy

Both ^1H and ^{13}C spectra were recorded on a Bruker AMX 500FT spectrometer (500 MHz, ^1H; 125 MHz, ^{13}C) (Billerica, MA, USA). MP-A40 was dissolved in deuterium oxide (D_2O, 3%, w/v) at room temperature for 3 h and freeze dried. This procedure was repeated three times before NMR analysis. The spectra of ^1H, ^{13}C, Correlated spectroscopy (COSY), Total Correlated Spectroscopy (TOCSY), Heteronuclear Multiple Quantum coherence spectroscopy (HMQC), Heteronuclear Multiple Bond Correlation spectroscopy (HMBC) and Nuclear Overhauser Effect spectroscopy (NOESY) of MP-A40 were all conducted at 60 °C.

2.6. Biological assays

2.6.1. Cell lines and culture

Human leukemia cell line K562 and RAW 264.7, a mouse macrophage cell line, were all purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). K562 cells were cultured in Gibco® RPMI Media 1640 (Life Technologies, NY, USA) containing 10% fetal bovine serum (FBS) (Life Technologies, NY, USA), 100 U/mL penicillin (Sigma–Aldrich, St. Louis, MO, USA) and

100 μg/mL streptomycin (Sigma–Aldrich, St. Louis, MO, USA) at 37 °C in a humidified atmosphere of 5% CO_2. RAW 264.7 cells were cultured at the same condition except Gibco® RPMI Media 1640 was replaced with Dulbecco's modified eagle medium (DEME, glutamine, high glucose, Gibco, USA). 5-Fluorouracil (5-FU) and lipopolysaccharides (LPS, from *Escherichia coli* serotype O111:B4) were from Sigma–Aldrich.

2.6.2. Inhibition of tumor cell proliferation in vitro

The inhibition effects of MP-A40 on K562 cells proliferation were determined *in vitro* using the Alamar Blue Assay (Ye et al., 2008). The cell suspension (2×10^4 cells/mL) was seeded into 96-Well plate (180 μL/well) and incubated. After 2 h, 20 μL of each sample was added into each well with different concentrations (10, 50, 200 and 500 μg/mL) and the microplates were incubated for another 48 h. PBS instead of samples served as negative control, and 5-FU (5 μg/mL) was as positive control. 5-FU is an antimetabolite that is used as a chemotherapeutic agent for a wide variety of cancers over 40 years. The antitumor activity mechanism of 5-FU was known as to affect the cell cycle and to induce cell apoptotic death (Li F. et al., 2008).

After 48 h, the absorbance was measured as the background value using a microplate reader at both 570 and 600 nm. Then 30 μL of Resazurin reagent (Biosource, Nivelles, Belgium) were added to each well and incubated for another 4 h. The absorbance was measured again at 570 nm and 600 nm. All determinations were done in triplicate. Inhibition ratio of tumor cell proliferation was calculated according to the equation below (Zhou et al., 2004):

$$\text{Inhibition rate}(\%) = \left\{1 - \frac{80,586 \times A_{570}(\text{sample}) - 117,216 \times A_{600}(\text{sample})}{80,586 \times A_{570}(\text{control}) - 117,216 \times A_{600}(\text{control})}\right\} \times 100\% \quad (1)$$

2.6.3. Effects of MP-A40 on NO production

180 μL of RAW 264.7 cells were incubated in each well of 96-Well plate at a density of 5×10^5 cells/mL for 2 h before adding another 20 μL of medium alone (negative control) or medium containing various concentrations of MP-A40 (10, 50, 100, 200 and 500 μg/mL) or LPS (1 μg/mL). After co-incubation for another 48 h, supernatants were collected for NO determination.

The accumulation of nitrite in the supernatants, which was used as an indicator of NO production in the medium, was measured by mixing 100 μL supernatants with 100 μL Griess reagent (1% sulfanilamide in 2.5% phosphoric acid, 0.1% N-[1-naphthyl]-ethylenediamine dihydrochloride in 5% phosphoric acid) and incubating at room temperature for 10 min. Nitrite production was determined by measuring absorbance at 540 nm *versus* a standard curve of $NaNO_2$ (Da Silva et al., 2009).

2.6.4. The effects of polymyxin B (PMB) on the NO production in RAW264.7 cells

Briefly, PMB was respectively mixed with MP-A40 or LPS to a final concentration 10 μg/mL, and incubated at room temperature for 2 h (Morrison & Jacobs, 1976). 20 μL of the resultant mixtures were added into 180 μL of RAW 264.7 cells (5×10^5 cells/mL) and incubated at 37 °C in a incubator with 5% CO_2 for another 48 h. Final concentrations of MP-A40 were 50 and 100 μg/mL, and 1 μg/mL for LPS. PBS was used as negative control. Supernatants were collected for NO determination as described above.

2.7. Statistical analysis

The results were presented as mean ± standard deviation (SD) of three independent determinations. All data were subjected to one-way analysis of variance (ANOVA) using SPSS statistical software (version 11.5 for Windows, SPSS Inc., Chicago, IL, USA). *P*-values of less than 0.05 were considered statistically significant.

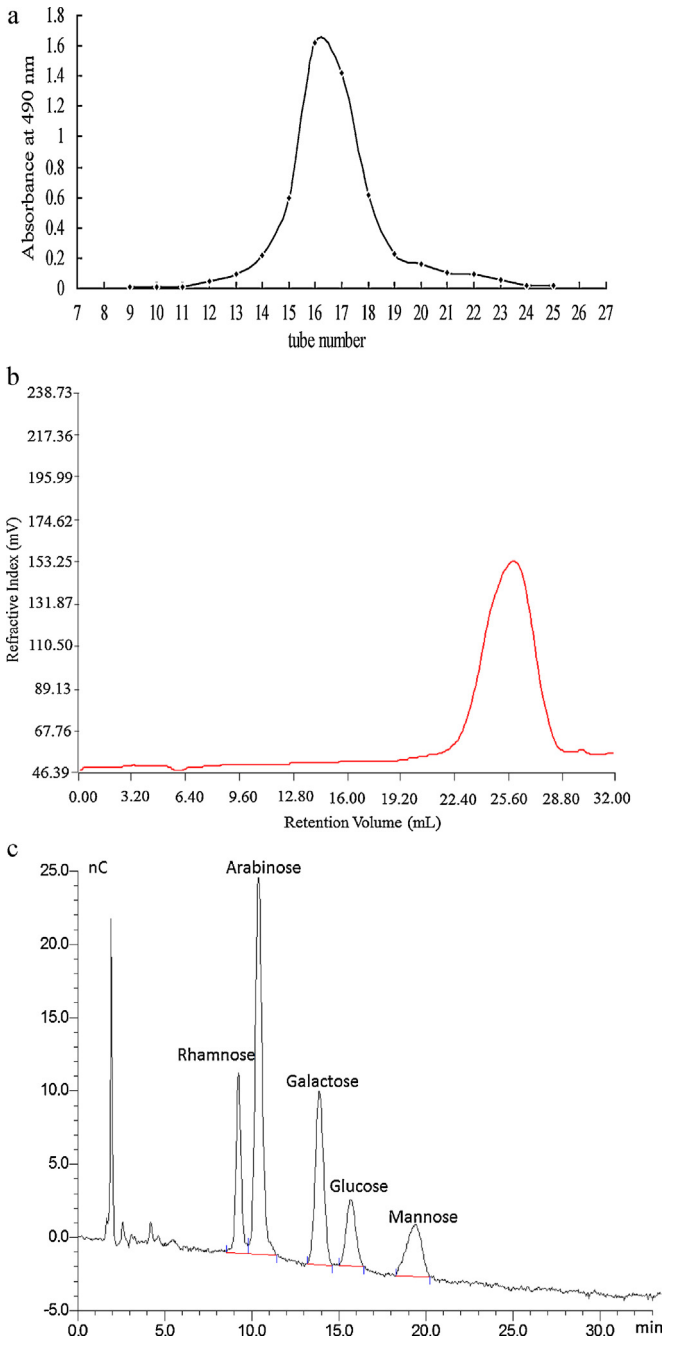

Fig. 1. Purification of MP-A40. (a) Elution profiles of MP-A40 from Q Sepharose™ Fast Flow column chromatography at 490 nm. (b) HPSEC profile of MP-A40 detected by refractive index (RI) detector. (c) HPAEC profile of monosaccharide composition of MP-A40.

3. Results and discussion

3.1. Isolation and purification and chemical characterization

Crude polysaccharides were obtained form the hot-water extract, and purified by a pre-equilibrated Q Sepharose™ Fast Flow ion exchange column and gradient ethanol precipitation method. The elution profile of MP-A40 (Fig. 1a) was determined by phenol-sulfuric acid method (Dubois et al., 1956). HPSEC was employed to

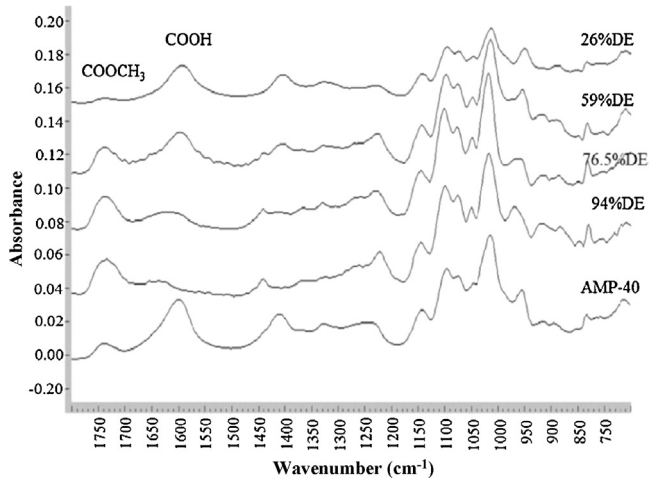

Fig. 2. Fourier transform infrared spectra of commercial pectin standards and MP-A40.

determine the homogeneity of MP-A40 which exhibited a single, symmetric peak in the RI profile (Fig. 1b).

Contents of neutral sugar, uronic acid and protein were determined to be 10.30 ± 0.04%, 68.63 ± 0.06%, and 0.17 ± 0.00%, respectively.

The average molecular weight of MP-A40 was estimated to be 32,600 Da. Monosaccharide composition analysis (Fig. 1c) showed that the neutral sugar of MP-A40 consisted of arabinose (4.94%), galactose (3.07%), rhamnose (2.13%), mannose (1.62%) and glucose (1.29%).

The degree of esterification was determined by FT-IR spectroscopic method. In Fig. 2, it was observed that the intensity of the absorbance or band area of the ester carbonyl groups (1760–1730 cm^{-1}) increased with the increase of DE of standard samples. In a similar manner, the intensity of the absorbance or band area of the free carboxylate groups (1630–1600 cm^{-1}) increased with the decrease of DE.

In order to quantify the DE of MP-A40, a calibration curve was constructed based on pectin standards with known DE values. The calibration curve y (%) = 81.525*x + 23.402, (R^2 = 0.9933), was established from the degree of esterification (y) and ratio of $A_{1730}/(A_{1730} + A_{1600})$ (x). DE of MP-A40 was calculated as 32.16%.

3.2. Methylation analysis of MP-A40

According to the retention time, characteristic ion fragments in MS spectra, combined with literature values as well (Sassaki, Gorin, Souza, Czelusniak, & Iacomini, 2005; Wang, He, & Huang, 2007), the linkage patterns of MP-A40 were summarized in Table 1, and the molar ratio of each sugar residue was calculated from the peak area in the total ion current (TIC) chromatogram.

The results indicated that MP-A40 was a polysaccharide with at least two kinds of branching points. The major terminal units were t-Gal*p*A (10.15%), with small amount of t-Ara*f* (0.60%) and t-Rha*p* (1.67%). The ratio of terminal units *versus* branching point was 1.45.

Table 1
Methylation analysis of the derivatives of MP-A40.

Retention time (min)	Sugar derivative	Linkage pattern	Molar ratio (%)
11.189	2,3,4-Me_3-Ara*f*	t-Ara*f*	0.60
12.642	2,3,4-Me_3-Rha*p*	t-Rha*p*	1.67
17.514	2,3,4,6-Me_4-Gal*p*A	t-Gal*p*A	10.15
23.342	2,3,6-Me_3-Gal*p*A	4-Gal*p*A	71.56
26.564	2,6-Me_2-Gal*p*A	3,4-Gal*p*A	4.29
28.515	3,6-Me_2-Gal*p*A	2,4-Gal*p*A	1.93

The data from the methylation analysis suggested that MP-A40 was mainly composed of →4Gal*p*A→1 as backbone (71.56%) with a few 3,4-Gal*p*A (4.29%) and 2,4-Gal*p*A (1.93%) providing branching points of the backbone chain at *O*-2 and *O*-3 positions with t-Gal*p*A, t-Ara*f* or t-Rha*p* as terminals.

3.3. NMR analysis

The structural features of MP-A40 were further elucidated by 1D/2D NMR analysis. The ^{1}H NMR (500.13 MHz) spectrum (Fig. 3a) of MP-A40 at 60 °C showed two anomeric proton signals at δ 4.983 and 4.794 ppm which were designated A and B, respectively. All the ^{1}H and ^{13}C signals were assigned using COSY (Fig. 4a), TOCSY (Fig. 4b), HMQC (Fig. 4c), HMBC (Fig. 4d) and NOESY (Fig. 4e).

The anomeric signals of residue **A** at 4.983 ppm (Fig. 3a) and 99.74 ppm (Fig. 3b) corresponded to an α-linked residue with a relatively high content in MP-A40. The proton assignment of residue A (from H-1 to H-5, 4.983, 3.634, 3.852, 4.344 and 4.953 ppm) was obtained from COSY spectrum (Fig. 4a). This assignment was also supported by the well resolved cross peaks in TOCSY spectrum (Fig. 4b). The corresponding chemical shifts of ^{13}C, revealed by HMQC spectrum (Fig. 4c), were 99.7, 69.1, 69.8, 79.3, 71.3 ppm for C-1, C-2, C-3, C-4 and C-5, respectively. The chemical shift of C-6 were confirmed by the intra-correlation cross peaks of residue **A** in HMBC spectrum (Fig. 4d), in which the cross peaks of H-5 with C-6 were clearly observed. According to Chandra, Ghosh, Ojha, and Islam (2009) and Sarkar et al. (2009), residue A was assigned as →4)-α-D-Gal*p*A-(1→ (**A**) after combining all the ^{1}H and ^{13}C chemical shifts, especially C-6 (171.8 ppm).

The anomeric proton assignment of residue **B** was at δ 4.794 ppm. The cross-peak of H-1/H-2 (4.794/3.624), H-2/H-3 (3.624/3.849), H-3/H-4 (3.849/4.293), H-4/H-5 (4.293/4.527) were assigned in the COSY spectrum (Fig. 4a) indicated that 3.624, 3.849, 4.293 and 4.527 ppm belonged to H-2, H-3, H-4 and H-5, respectively. Chemical shifts from H-2 to H-5 can also be determined from the TOCSY (Fig. 4b).

On the basis of the proton assignments, the chemical shifts of C-1 to C-5 were obtained from the HMQC spectrum (Fig. 4c) (Ovodova et al., 2009). A cross peaks (4.527/176.1) between H-5 and C-6 of residue **B** were observed in HMBC spectrum (Fig. 4d). There was an obvious cross peak with the proton chemical shift of 3.694 ppm on HMQC (Fig. 4c). This peak was not derived from the sugar ring of residue **B**. By comparing with literature data (Ghosh, Chandra, Ojha, Sarkar, & Islam, 2009; Mondal, Das, Maiti, Roy, & Islam, 2009), this peak was assigned to the proton (3.694 ppm) and carbon (53.75 ppm) of methoxy group. The results indicated that residue **B** was present as →4)-α-D-GalpA6Me-(1→ (**B**).

The sequence of glycosyl resiudes of MP-A40 was also confirmed by NOESY experiment (Fig. 4e and Table 2). In NOESY experiment, the intra-residual cross peaks were observed from **A** H-1 to **A** H-2, **A** H-3, **A** H-4, **A** H-5; **A** H-2 to **A** H-3, **A** H-4, **A** H-5; **A** H-3 to **A** H-4, **A** H-5; **A** H-4 to **A** H-5; **B** H-1 to **B** H-2, **B** H-3, **B** H-4, **B** H-5; **B** H-2 to **B** H-3, **B** H-4, **B** H-5; **B** H-3 to **B** H-4, **B** H-5; **B** H-4 to **B** H-5, along with inter-residual contacts from **A** H-1 to **B** H-1, **B** H-2, **B** H-3, **B** H-4, **B** H-5; **A** H-2 to **B** H-1, **B** H-3, **B** H-4, **B** H-5; **A** H-3 to **B** H-1, **B** H-2, **B** H-4, **B** H-5; **A** H-4 to **B** H-1, **B** H-2, **B** H-3, **B** H-5.

According to above analysis, all the chemical shifts of residue **A** and **B** were identified. The complete assignments are presented in Table 3. The major repeating unit of the pectic polysaccharide (MP-A40) was established as →4)-[α-Gal*p*A6Me-(1→]$_m$-[4-α-Gal*p*A-(1→]$_n$.

3.4. Inhibition of tumor cell proliferation in vitro

In the past few years, studies related with the effects of polysaccharides on the tumor cells have increased. A polysaccharide

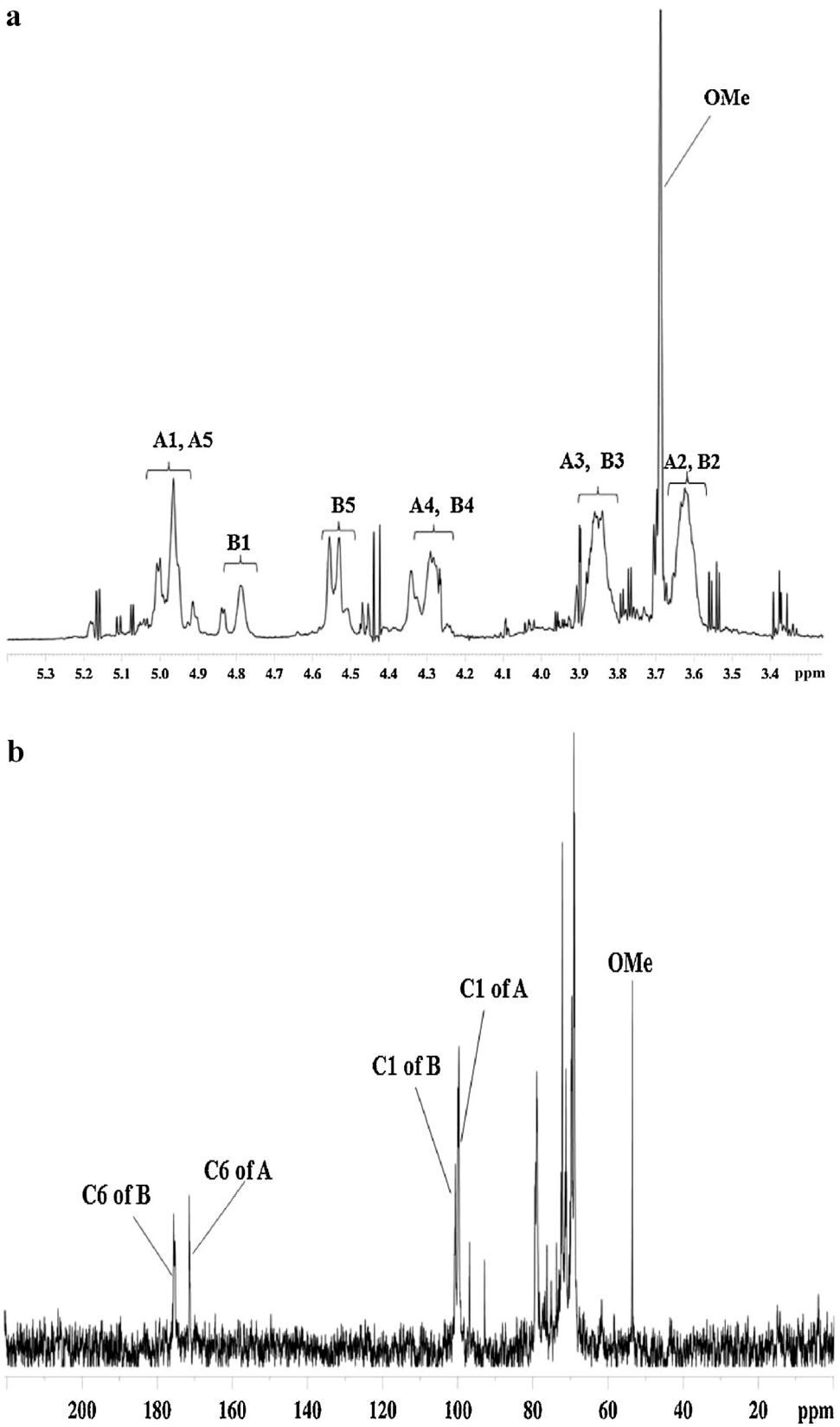

Fig. 3. 1D NMR spectrum of polysaccharide MP-A40 isolated from *Mosla chinensis* Maxim. cv. Jiangxiangru. (a) ^{1}H NMR spectrum (60 °C in D_2O, 500.13 MHz). (b) ^{13}C NMR spectrum (60 °C in D_2O, 125.78 MHz).

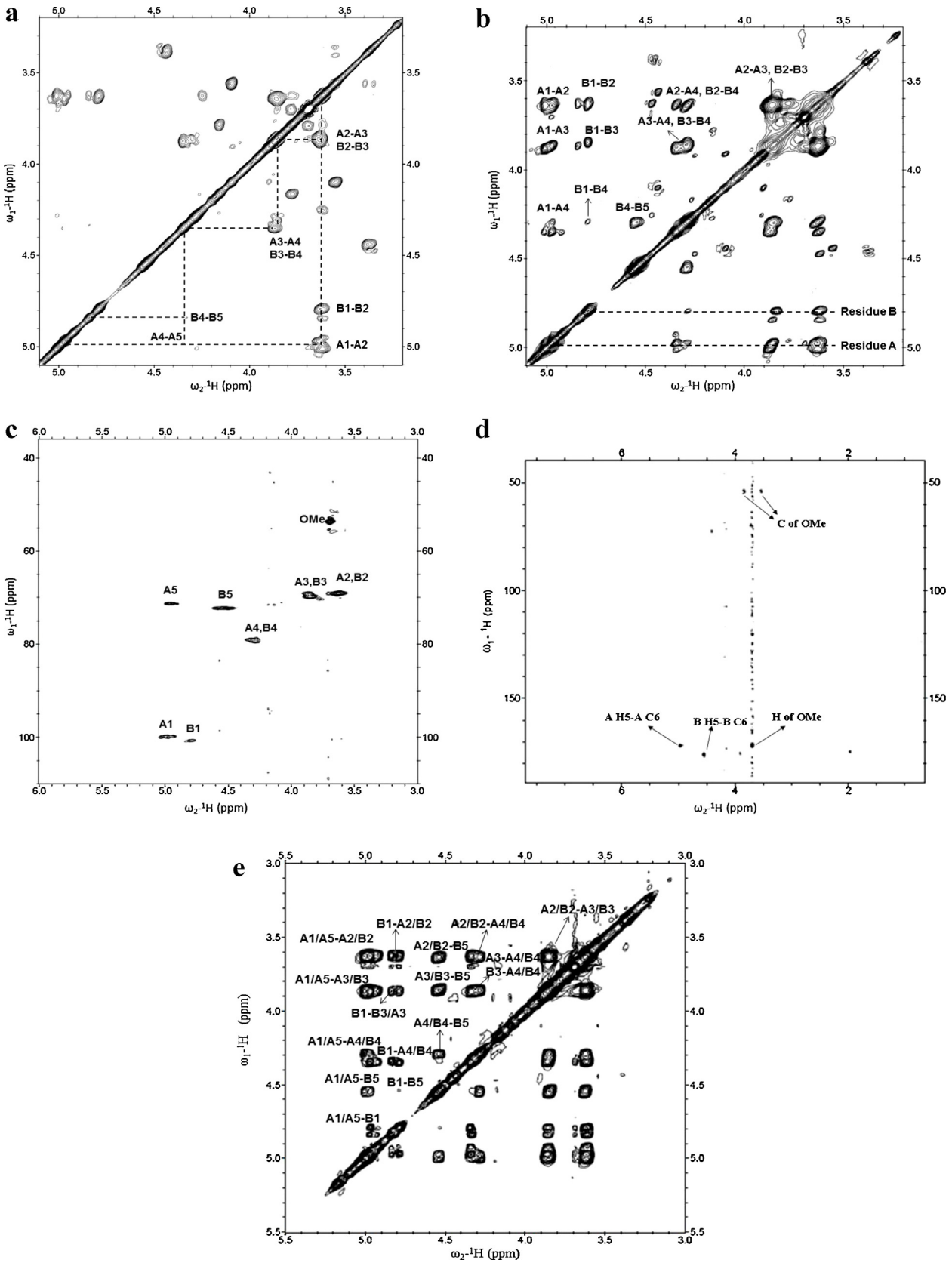

Fig. 4. 2D NMR spectrum of polysaccharide MP-A40 isolated from *Mosla chinensis* Maxim. cv. Jiangxiangru. (a) COSY spectrum (60 °C in D_2O). (b) TOCSY spectrum (60 °C in D_2O). (c) HMQC spectrum (60 °C in D_2O). (d) HMBC spectrum (60 °C in D_2O). (e) NOESY spectrum (60 °C in D_2O).

Table 2
Assignment of cross-peaks in the NOESY spectrum of MP-A40.

Residue	Proton	δ H (ppm)	Observed connectivities			
			Intra		Inter	
			Residue, proton	δ H (ppm)	Residue, proton	δ H (ppm)
→4)- α-D-Gal*p*A-(1→ **A**	H-1	4.930	**A** H-2	3.647	**B** H-1	4.796
			A H-3	3.876	**B** H-2	3.630
			A H-4	4.321	**B** H-3	3.864
			A H-5	4.968	**B** H-4	4.308
					B H-5	4.551
	H-2	3.634	**A** H-3	3.853	**B** H-1	4.713
			A H-4	4.326	**B** H-3	3.853
			A H-5	4.972	**B** H-4	4.319
					B H-5	4.551
	H-3	3.852	**A** H-4	4.332	**B** H-1	4.795
			A H-5	4.967	**B** H-2	3.640
					B H-3	3.855
					B H-4	4.312
					B H-5	4.541
	H-4	4.344	**A** H-5	4.965	**B** H-1	4.807
					B H-2	3.632
					B H-3	3.862
					B H-4	4.316
					B H-5	4.534
→4)-α-D-Gal*p*A6Me-(1→ **B**	H-1	4.794	**B** H-2	3.622		
			B H-3	3.865		
			B H-4	4.312		
			B H-5	4.537		
	H-2	3.639	**B** H-3	3.868		
			B H-4	4.315		
			B H-5	4.532		
	H-3	3.863	**B** H-4	4.317		
			B H-5	4.533		
	H-4	4.295	**B** H-5	4.538		

(SMP-W1) from *Salvia miltiorrhiza* significantly inhibited the growth of hepatocellular carcinoma H22 cells by 40% at 400 μg/mL (Liu L. et al., 2013). Another study by Liu Y. et al. (2012), indicated that ginseng polysaccharide (GPS) has an inhibitory rate of 43.85 ± 0.87% to K562 cells at 400 μg/mL after incubation for 48 h. It has been reported that polysaccharides have different antitumor activities *in vitro*, depending on their monosaccharide composition, protein contents, molecular mass, and chain conformation (Cui F.J. et al., 2007).

In this study, when K562 cells were treated with MP-A40 (10, 50, 200 and 500 μg/mL) or 5-FU (5 μg/mL) and incubated for 48 h, the growth of tumor cells were inhibited at different levels. From Fig. 5a, the inhibition ratio of K562 cells was 4.32% at 10 μg/mL, and a higher inhibition ratio of 31.32% was observed at 500 μg/mL, which were lower than that of 5-FU (65.54%) at 5 μg/mL. The result showed MP-A40 inhibited the proliferation of K562 cells in a dose-dependent manner.

3.5. Effects of MP-A40 on NO production

Macrophage is the most important professional phagocyte and it plays an essential and pivotal role in host defense against any type of invading cells including tumor cells (Katsiari, Liossis, & Sfikakis, 2010). NO has been identified as a major effector molecule produced by macrophages and has been involved both in the regulation of apoptosis and in host defenses against microorganisms and tumor cells (Brüne, 2003). A polysaccharide fraction, J6, from hot-water extract of oleander *Nerium indicum* Mill. flower, stimulated NO production of macrophage RAW 264.7 cells from 21 (31.25 μg/mL) to 36 μM (500 μg/mL) in a dose-dependent manner (Dong et al., 2010).

In our study, macrophages were incubated with MP-A40 of different concentrations from 10 to 500 μg/mL for 48 h. As shown in Fig. 5b, a minimum amount of NO was produced when RAW 264.7 cells were incubated with medium alone; whereas, treatment with MP-A40 resulted in a dose-dependent increase in NO production and up to maximum of 35.02 μM at 500 μg/mL. When the concentration of MP-A40 was as low as 10 μg/mL, the NO production was approximately 15 times of negative control. While from 200 to 500 μg/mL, there was no significant increase of NO production (from 32.58 to 35.02 μM). The result demonstrated MP-A40 had a strong effect on macrophages activation.

3.6. The effects of polymyxin B (PMB) on the NO production in RAW264.7 cells

Polysaccharide samples isolated from plant sources are often contaminated with endotoxin. Potential contamination of endotoxin is always a concern for the high-molecular-weight components isolated from plants, which may contributes to their

Table 3
^{1}H NMR and ^{13}C NMR chemical shifts of the MP-A40 recorded in D_2O at 60 °C.

Residue	H-1 C-1	H-2 C-2	H-3 C-3	H-4 C-4	H-5 C-5	H-6a, 6b C-6	OMe
→4)-α-D-Gal*p*A-(1→ **A**	4.983	3.634	3.852	4.344	4.953		
	99.7	69.1	69.8	79.3	71.3	171.8	
→4)-α-D-Gal*p*A6Me-(1→ **B**	4.794	3.624	3.849	4.293	4.527		3.694
	100.7	69.1	69.8	79.3	72.3	176.1	53.75

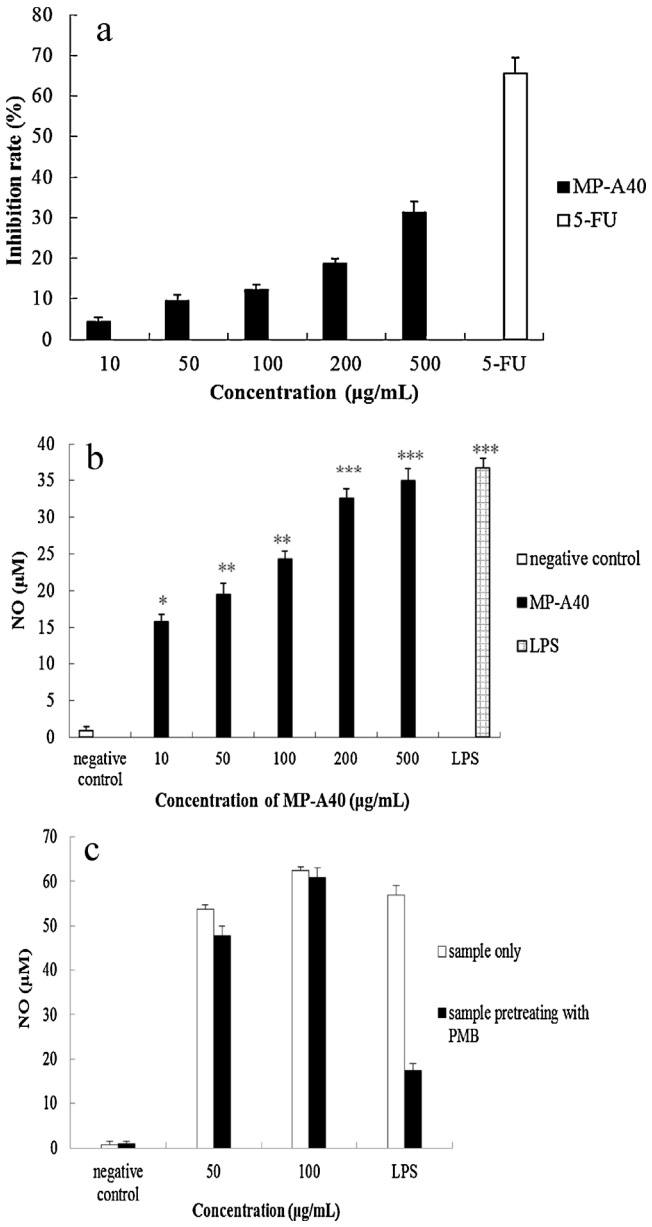

Fig. 5. Bioactivities of MP-A40. (a) Effect of MP-A40 on K562 tumor cells proliferation *in vitro*. (The results were expressed as mean values ± standard deviations.) (b) Effect of MP-A40 on the NO production in RAW 264.7 cells. Significances were determined using the ANOVA test *versus* control group (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). (c) Effect of MP-A40 on NO production in RAW 264.7 cells after pretreating with polymyxin B (PMB).

bioactivities (Chen, Meng, Liu, Chen, & Zhang, 2010). Lipopolysaccharide (LPS) is a common endotoxin from the cell wall of Gram-negative bacteria that can exhibit various immunological activities, including NO-inducing effect, thus LPS contamination may lead to a misunderstanding on the biological test result. Therefore, a number of approaches were carried out to prevent possible LPS contamination (Schepetkin, Faulkner, Nelson-Overton, Wiley, & Quinn, 2005).

In this study, antibiotic polymyxin B, which could inactivate endotoxin by high affinity to lipid A of LPS, was used to investigate whether samples was contaminated with endotoxin.

In this study, the effects of PMB treatment on the NO production of MP-A40 or LPS were examined. Fig. 5c showed that with 10 μg/mL PMB addition, NO production did not significantly change when co-incubating with MP-A40 from 50 to 100 μg/mL ($P > 0.05$), however, LPS-induced NO production was significantly decreased. These results indicated that the effect of MP-A40 on the NO production was not due to endotoxin contamination.

4. Conclusions

This is the first report about the structure characterization of a pectic polysaccharide isolated from *M. chinensis* Maxim. cv. Jiangxiangru. In this study, MP-A40 exhibited clear antitumor activity *in vitro* on human leukemia cell line K562, and it also enhanced the NO production from RAW 264.7 macrophages in a dose-dependent manner, indicating a potential pharmaceutical value of this plant. Further studies need to be carried on to elucidate the possible mechanisms of antitumor and immune modulation, and the relationship between structure and function of MP-A40.

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