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# Pectic oligosaccharides hydrolyzed from citrus canning processing water by Fenton reaction and their antiproliferation potentials

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## ABSTRACT

Citrus canning processing water contains a valuable and renewable source of biopolymers and bioactive compounds including pectic polysaccharides. Upgrading these processing wastes can not only alleviate environmental pollution but also add value to the commodity's production. In a previous study we recovered pectic polysaccharides from citrus canning processing water. In the present study, pectic polysaccharides recycled from citrus canning processing water was depolymerized by an optimized Fenton system. The hydrolyzate was fractionated via size-exclusion chromatography into six fractions: 500 Da < LMP1 < 3 kDa; 3 kDa < LMP2 < 5 kDa; 5 kDa < LMP3 < 12 kDa; 12 kDa < LMP4 < 25 kDa; 25 kDa < LMP5 < 100 kDa and LMP6 > 10 wDa. Structure analyses showed that LMP1 were homogalacturonans-enriched non-esterified polysaccharides. While LMP2 contained both HG and rhamnogalacturonan-I (RG-I). Further antitumor assay showed that in comparison with the native pectic polysaccharide with moderate antitumor activity, both LMP1 and LMP2 possessed significant antitumor activity, while the inhibitory effect of LMP1 was higher than that of LMP2, suggesting that the biological properties of LMPs was influenced by structural characteristics, including molecular weight and monosaccharide composition.

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## 1. Introduction

Canned citrus segments are popular all over the world because they are a delicious and convenient fruit product, and China occupies the nearly 70% of the canned citrus segments on the international market [1]. However, removing membranes from the segments, which is a critical step in canning production, produces a large quantity of liquid waste (mainly polysaccharides) with high chemical oxygen demand (COD), posing serious environmental issues and creating resource waste [2]. Therefore, converting these liquid wastes into energy or other chemicals that are used in our daily activities is an area of research with huge potential and opportunities.

In a previous study, we demonstrated that the organic substances in the processing water from citrus canning factories are principally pectic polysaccharides (PPs) [2]. Pectic polysaccharides are widely used in the food industry as thickeners and gelling agents [3] and exhibit a broad range of pharmacologic properties, including antitumor [4,5], antioxidant [6], immunomodulatory and gastroprotective activities [7]. The functionalities of pectic polysaccharides are closely associated with

their structures including the contents in GalA and neutral sugars, the amounts and distributions of substituents (methoxyl and acetyl groups) and molecular weight and its distribution [8]. Generally, pectic polysaccharides that are dominated by homogalacturonans (HG) regions possess greater potential in gel applications, while highly branched pectic polysaccharides enriched with an RG-I region show better application for thickening and emulsifying [2]. The immunomodulatory activity of pectic polysaccharides is attributed to the presence of immune active arabinogalactan (AG) type I and II structures O-4 attached to the Rha of the rhamnogalacturonan-I (RG-I) domain [9,10]. The RG-I enriched PPs are known for their significant antitumor activity as a galectin 3 inhibitory agent [11]. Therefore, identifying the structural properties of recovered pectic polysaccharides from citrus canning factories helps us better comprehend and predict the PPs' potential in biological activity and gelling properties [12].

Pectic polysaccharides are a family of complex heteropolysaccharides. Although the structures of PPs that were recycled from citrus canning processing water were preliminarily identified by FT-IR spectra and composition analysis in our previous study [2], the delicate structural properties of PPs was still unclear due to their high molecular weight, complex composition and the difficulty of obtaining a purified polysaccharide. The degradation of native polysaccharides to relatively low molecular weight fragments, which retains the primary structure of the native

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polysaccharides, has been the typical strategy for analysing native polysaccharide [13,14]. Additionally, in comparison with native pectic polysaccharides, low molecular weight pectic polysaccharides (LMP) have improved absorption rate [11] and higher biological activity [5,12,15].

Controlled chemical or enzymatic depolymerization processes have been widely used to prepare low molecular weight pectic polysaccharides. Chemical methods that rely on acid-catalysed hydrolysis can result in the hydrolysis of acid-labile linkages between the galacturonic acid (GalA) and rhamnose (Rha) residues in the RG-I region [16] and the concomitant decomposition of sugars to furyl aldehydes and other side products, which decreases the yield and results in toxic components [12]. The enzymatic hydrolysis of pectic polysaccharides is highly specific for cleaving glycosidic bonds in the polysaccharide chain. However, enzymatic depolymerisation methods require the use of different types of enzymes and may be at risk of microbial contamination of LMP preparations [17]. Controlled oxidative depolymerization of polysaccharides usually utilizes hydrogen peroxide-dependent systems to generate reactive oxygen species including HO•. These radicals depolymerize the polysaccharides by attacking and breaking the glycosidic linkages without obvious structural changes to the sugar units [5,18]. Therefore, radical depolymerisation is available for the preparation of LMPs, and the structure characteristics of native polysaccharides can be inferred at the same time.

In the present study, we apply a controlled Fenton system to obtain LMPs, and factors affecting LMP yields are investigated. The resulting fragments were further fractionated and the structural properties of these obtained fractions were characterized by monosaccharide composition analysis, Fourier transform-infrared (FT-IR), nuclear magnetic resonance (NMR) spectroscopy and HILIC-MS. Moreover, the *in vitro* tumour cell growth inhibitory effects of native polysaccharides and LMPs were evaluated on MCF-7 human breast adenocarcinoma cells using a 3 (4,5 dimethylthiazol 2 yl) 2,5 diphenyltetrazolium bromide (MTT) assay.

## 2. Materials and methods

### 2.1. Materials

We collected the water discharged from citrus canning factories during the segment membrane removal process (Ningbo, China). The Ultrahydrogel 250 gel-filtration column and TSK-Gel G 4000 SWXL columns were from Waters and Tosoh Biosep, respectively. Hydrogen peroxide, copper (II) acetate and HPLC-grade methyl alcohol were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). The 95% (v/v) ethanol (food grade) and other chemical reagents were acquired from Aladdin Chemical Reagent Co., Ltd. (Shanghai, China).

### 2.2. Optimization of degradation by the Fenton system

A solution of native pectic polysaccharide (0.5% m/v) was added to a solution of H<sub>2</sub>O<sub>2</sub> and Cu<sup>2+</sup> according to the group arrangements in Table 1. The sample solution was stirred and reacted at room temperature. Sodium hydroxide was added to terminate the reaction. The depolymerized products were desalinated by dialysis with a 500 Da cut-off membrane for 72 h, concentrated and subsequently lyophilized.

### 2.3. Fractionation of depolymerized pectic polysaccharides

The resulting mixture was fractionated by gel filtration chromatography in a Superdex 30 column (5 × 200 cm) that was eluted with 0.3 M NH<sub>4</sub>HCO<sub>3</sub> for 500 min at a flow rate of 0.5 mL/min. Carbohydrate fractions were detected by high performance size exclusion chromatography (HPSEC) as previously described by Chen et al. [19]. The pooled oligosaccharide fractions were further analysed by high performance size exclusion chromatography (HPSEC) and were then lyophilized.

**Table 1**  
Effect of the concentrations of H<sub>2</sub>O<sub>2</sub> and Cu<sup>2+</sup> and the reaction time on the yields of LMPs.

Samples number	Reaction conditions			
	H <sub>2</sub> O <sub>2</sub> (%)	Cu <sup>2+</sup> (mM)	Reaction time (h)	Peak area ratio of LMP <sup>a</sup> (%)
Group 1	1	1.5	0.5	19.57
	1	1.5	1	23.45
	1	1.5	2	26.63
	1	1.5	3	58.18
Group 2	1	3	0.5	25.85
	1	3	1	44.26
	1	3	2	52.85
	1	3	3	50.16
Group 3	2	1.5	0.5	20.03
	2	1.5	1	22.61
	2	1.5	2	23.79
	2	1.5	3	51.11
Group 4	2	3	0.5	47.78
	2	3	1	51.28
	2	3	2	54.47
	2	3	3	56.03
Group 5	4	1.5	0.5	40.53
	4	1.5	1	47.55
	4	1.5	2	48.36
	4	1.5	3	55.67
Group 6	4	3	0.5	56.47
	4	3	1	57.17
	4	3	2	57.85
	4	3	3	57.86

<sup>a</sup> Stands for the low molecular weight pectic polysaccharides with molecular weights below 5 kDa.

### 2.4. Determination of monosaccharide composition

The monosaccharide composition of the oligosaccharide fragments was determined by the 1 phenyl 3 methyl 5 pyrazolone (PMP) high performance liquid chromatography (HPLC) method [5]. Briefly, approximately 2 mg of the pectin samples were hydrolyzed with 4 M trifluoroacetic acid (TFA) at 110 °C for 8 h. After cooling to room temperature, the TFA was removed and the reaction solution was adjusted to a pH of 7.0 with 2 M NaOH, and then with 0.3 M NaOH. The hydrolysate was derivatized with 50 µL of 0.3 M NaOH and 50 µL of 0.5 M PMP solution at 70 °C for 100 min. Chloroform was used to extract the hydrolysate, then the hydrolysate was analysed by a Waters 2695 HPLC system (Waters, USA) with an ZORBAX Eclipse XDB-C18 column (Agilent, 5 µm, 4.6 mm × 250 mm, Santa Clara, CA, USA). Mobile phase A was aqueous and contained sodium phosphate buffer (0.05 M, pH 6.9) and acetonitrile (v/v; 85:15), and mobile phase B was aqueous and contained sodium phosphate buffer (0.05 M, pH 6.9) and acetonitrile (v/v; 60:40). The time program of the HPLC analysis was 0 → 10 → 30 min, the concentration program of mobile phase B was 0 → 8% → 20% at a flow rate of 1 mL/min, the samples were detected by UV detection at 250 nm and the injection volume was 20 µL.

### 2.5. IR spectral analysis

The FT-IR analysis was applied to obtain the IR spectra of the pectin samples using a Nicolet Avatar 370 instrument. Samples (~1 mg) were ground together with 200 mg of KBr and were pressed into pellets for 32 IR scans from 400 to 4000 cm<sup>-1</sup> at a 4 cm<sup>-1</sup> resolution.

### 2.6. NMR analysis of LMPs

For the NMR analysis, citrus pectin and LMP fractions (~5 mg) were evaporated twice with 550 µL of D<sub>2</sub>O (99.96%) by vacuum freeze drying before final dissolution in 550 µL of D<sub>2</sub>O (99.96%). NMR spectra were collected by a 600 MHz NMR spectrometer (DD2-600; Agilent Technologies Inc., CA, US) at 25 °C. The spectra were processed using the

MestReNova 6.1.1 (MestreLab Research, Santiago de Compostela, Spain). The number of scans (ns) in each experiment was dependent on the sample concentrations.

### 2.7. Top-down analysis of LMPs

The top-down analysis was executed according to a previously described method [20]. LMP1 and LMP2 were separated using a Luna HILIC column (2.0 × 150 mm<sup>2</sup>, 200 Å, Phenomenex, Torrance, CA). The mobile phases A and B were 5 mM ammonium acetate and water-acetonitrile (2/98, v/v), respectively. The gradient was performed from 5% A to 70% A over 7 min at a flow rate of 250 µL/min, and then it was reset to 5% A. The LC column was directly connected online to the standard ESI source LTQ-Orbitrap XL FT MS (Thermo Fisher Scientific, San Jose, CA). All of the parameters were optimized using Arixtra™ to decrease the in-source fragmentation, sulfate loss and the noise/signal in the negative-ion mode. The optimized source parameters were as follows: spray voltage = 4.2 kV, capillary voltage = −40 V, tube lens voltage = −50 V, capillary temperature = 275 °C, sheath flow rate = 30 L/min and auxiliary gas flow rate = 6 L/min. All of the FT mass spectra were obtained at a resolution of 60,000 with a 400–2000 Da mass range.

Charge deconvolution was conducted using DeconTools software. The structural assignments of the LMPs and the acquisition of a hypothesis database were both performed using GlycReSoft 1.0 software. All of the quantitative data was normalized to the total identified oligosaccharides peak area (%).

### 2.8. Cell viability assay

The antitumor activity of PPs and LMWP on MCF-7 cells was evaluated using a tetrazolium salt 3 (4,5 dimethylthiazol 2 yl) 2, 5 diphenyltetrazolium bromide (MTT) assay [21]. The cells were incubated in Dulbecco's modified eagle medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 100 U/mL of penicillin and 100 g/mL of streptomycin at 37 °C in a humidified incubator at 5% CO<sub>2</sub>. Briefly, 100 µL of the cells were incubated in a 96-well plate at a concentration of 2 × 10<sup>5</sup> cells/mL. After 24 h of cultivation, various concentrations of PPs and LMPs (0, 50, 125, 250, 500, 1000 and 2000 µg/mL) were added slowly into the 96-well plate and cultured for 48 h. At the end of each treatment, 20 µL of MTT (5 mg/mL) was added and the tumour cells were further incubated for 4 h to allow the formation of formazan crystals. A volume of 150 µL DMSO was added to each well to dissolve the formazan crystals after the medium was removed. Subsequently, absorbance was measured at 490 nm with a microplate reader (Thermo multiscan Mk3, Thermo Fisher Scientific Inc., USA). The cell viability was expressed as

$$\text{Cell viability (\%control)} = [(A_s - A_b)/(A_c - A_b)] \times 100$$

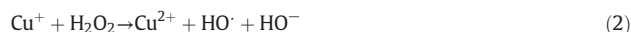
where  $A_c$  and  $A_b$  were the absorbance of the system without the addition of polysaccharides or LMPs, respectively, and  $A_s$  was the absorbance of the system only with polysaccharides or LMPs.

## 3. Results and discussion

### 3.1. Optimization of the degradation

To develop a more efficient method for the preparation of low molecular weight pectic polysaccharides, the effects of H<sub>2</sub>O<sub>2</sub> concentrations, Cu<sup>2+</sup> concentrations and reaction time on the yields of LMPs were estimated and we defined pectic polysaccharides with molecular weights below 5 kDa as target products. In the present study, the ratio of the LMP area to the total area of the resulting fragment in the GPC profile was selected as an index to reflect the yield of LMPs indirectly.

As presented in Table 1, the higher the concentration of Cu<sup>2+</sup> when the concentration of H<sub>2</sub>O<sub>2</sub> and the reaction time are fixed, the higher the yields of LMPs. In the Fenton system, hydrogen peroxide is activated by copper (Cu<sup>2+</sup>) ions to generate hydroxyl radicals (HO•) via a complex reaction sequence (Eqs. (1) and (2)) [22], and the unpaired electron of HO• radicals makes them strong oxidizing agents, which react with pectic polysaccharides and causes their depolymerisation. When there is an excess of H<sub>2</sub>O<sub>2</sub>, the increase of Cu<sup>2+</sup> as a catalyst can significantly accelerate the generation of hydroxyl radicals per unit time, thus elevating the yield of LMPs. Similarly, increasing H<sub>2</sub>O<sub>2</sub> concentration from 1% to 2% can also result in the higher LMP yields when there is mixed Cu<sup>2+</sup>. Nevertheless, when the H<sub>2</sub>O<sub>2</sub> concentration was further increased to 4%, no further obvious improvement for the LMP yields could be observed. The excess addition of H<sub>2</sub>O<sub>2</sub> could lead to the higher concentration of H<sup>+</sup> in the system, and the reaction between Cu<sup>+</sup> and H<sub>2</sub>O<sub>2</sub> (Eq. (1)) is severely inhibited by molecular oxygen since Cu<sup>+</sup> is quantitatively oxidized by oxygen to Cu<sup>2+</sup> in acidic conditions (Eq. (3)), reducing the effective [Cu<sup>+</sup>] available to react with H<sub>2</sub>O<sub>2</sub> and decreasing the process's efficiency [22]. Considering the degradation efficiency, yields and the cost, we selected 2% H<sub>2</sub>O<sub>2</sub>, 3 mM Cu<sup>2+</sup> and a reaction time of 2 h as the suitable values to prepare LMPs.

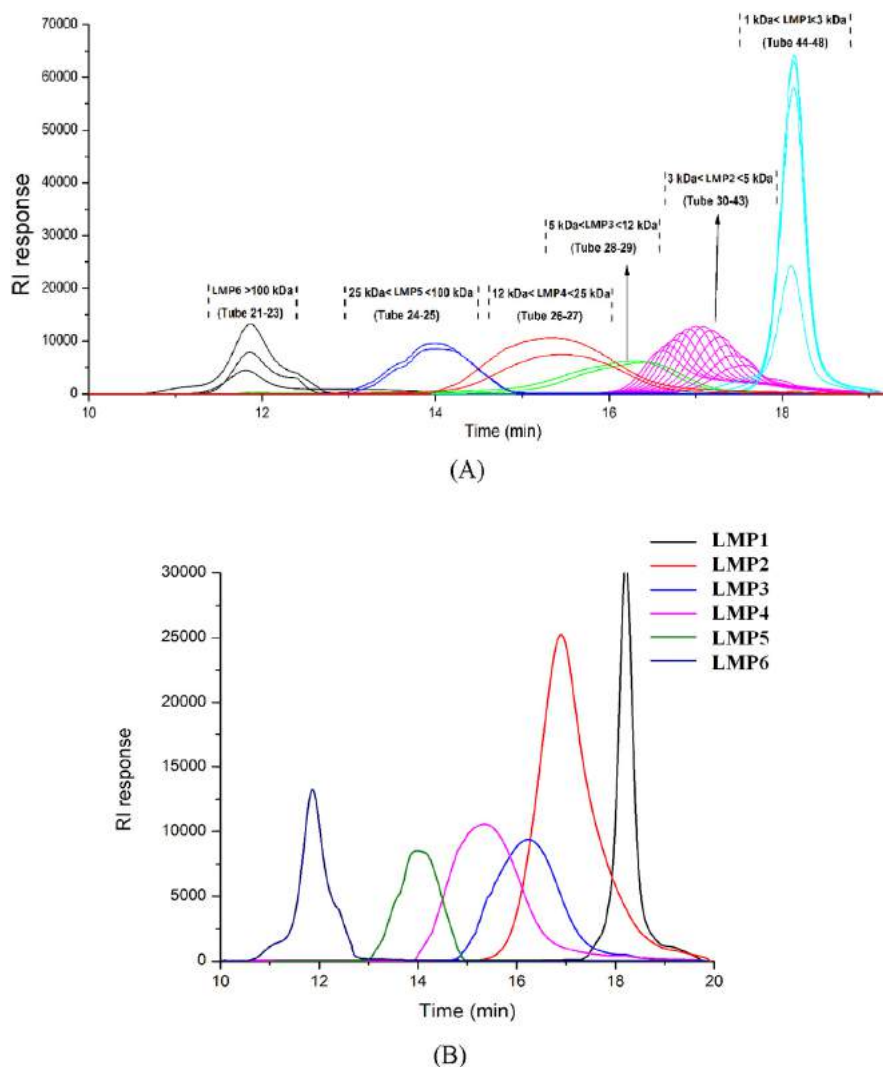


### 3.2. Preparation of LMP fractions

Native pectic polysaccharides were depolymerized under optimized conditions and produced a high yield of LMPs. The depolymerized PPs were size-fractionated by gel filtration chromatography in a Superdex 30 column and the samples obtained were analysed by high performance size exclusion chromatography (HPSEC) (Fig. 1A). Based on the molecular weight according to the HPSEC, we separated the depolymerized PPs into six fractions (LMP1–LMP6): 500 Da < LMP1 < 3 kDa; 3 kDa < LMP2 < 5 kDa; 5 kDa < LMP3 < 12 kDa; 12 kDa < LMP4 < 25 kDa; 25 kDa < LMP5 < 100 kDa and LMP6 > 100 kDa (Fig. 1B). The absolute yield from the PPs of LMP 1–6 was 26.3%, 31.8%, 10.2%, 7.9%, 7.2% and 8.4%, respectively. It has been demonstrated that rhamnogalacturonan I (RG I) elutes in a broad and wide molar mass range whereas homogalacturonans (HG) elutes in a single narrow and symmetrical peak as analysed by HPSEC [8]. Therefore, we concluded that LMP1 was rich in HG, while LMP2 contained RG I.

### 3.3. Chemical compositional analysis

The results that are presented in Table 2 indicated the presence of Man, Rha, GlcA, GalA, Glu, Gal, Ara, Xyl and Fuc in different proportions among the LMPs. Specifically, GalA (in mole %) was the principle component in both LMP1 and LMP2, while arabinose (Ara) was the predominant neutral saccharide followed by galactose (Gal) and glucose (Glc). However, in comparison with LMP1, LMP2 contains a higher arabinose content (35.21%) and galactose content (20.10%) and a lower galacturonic acid content (19.04%). Similar to LMP2, LMP3 also has relatively high arabinose content (35.55%). Interestingly, with the molecular weight increasing from LMP3–LMP6, the glucose content markedly increased while the arabinose content significant decreased. Judging by the Rha/GalA ratio [23], the high ratio of 7.56 indicated that LMP1 was relatively rich in homogalacturonans (HG) as opposed to rhamnogalacturonans (RG), while the low ratio of 2.68 showed that LMP2 was rich in both RG and HG. Previous studies have shown that OH•, which is the reactive species, primarily attacks the glycosidic bond, and the GalA residues on the HG domain are more reactive with hydroxyl radicals, resulting in chain scission



**Fig. 1.** Preparation of a series of LMPs by free radical depolymerization. (A) The HPSEC chromatography of isolated fractions from depolymerized PP by gel filtration chromatography; (B) the HPSEC chromatography of LMP1-LMP6 from PPs.

and RG-I enriched fragments [5,15]. As with the resulting fragments of PPs from free radical degradation, the high amount of HG in both LMP1 and LMP2 suggested that the native pectic polysaccharide from citrus canning processing water predominantly consists of HG, which is consistent with HPSEC chromatography and the report by Yapo et al. [8]. The ratio of (Ara + Gal)/Rha was calculated as an estimate of the relative importance

of the neutral side-chains to the rhamnogalacturonan backbone. The high ratio of (Ara + Gal)/Rha in both LMP1 and LMP2 suggested the presence of neutral side chains to the backbone of rhamnogalacturonan, such as arabinan, galactan, arabinogalactans and galactoarabinans. The results indicated that the structure and the composition of the pectic polysaccharides that are recycled from canning process water are very complex.

**Table 2**  
Monosaccharide composition of the six LMPs.

Monosaccharide (mol%)	LMP1	LMP2	LMP3	LMP4	LMP5	LMP6
Man	5.31 ± 0.03	1.84 ± 0.24	2.66 ± 0.27	3.35 ± 0.35	4.18 ± 0.35	2.17 ± 0.03
Rha	5.82 ± 0.04	7.10 ± 0.64	4.19 ± 0.39	3.98 ± 0.28	4.48 ± 0.28	4.71 ± 0.04
GlcA	0.50 ± 0.01	0.44 ± 0.01	1.00 ± 0.03	2.51 ± 0.17	2.65 ± 0.37	2.10 ± 0.02
GalA	44.15 ± 2.45	19.04 ± 1.04	6.35 ± 0.68	8.04 ± 0.84	8.83 ± 1.04	5.66 ± 0.04
Glu	10.08 ± 0.74	13.58 ± 0.83	33.28 ± 2.85	61.75 ± 4.06	69.33 ± 5.27	82.28 ± 6.89
Gal	12.92 ± 0.83	20.10 ± 1.85	15.00 ± 1.42	4.09 ± 0.42	2.56 ± 0.42	1.11 ± 0.03
Xyl	1.06 ± 0.02	2.08 ± 0.32	1.77 ± 0.03	0.36 ± 0.01	—	—
Ara	19.93 ± 1.02	35.21 ± 2.03	35.55 ± 3.4	14.00 ± 1.04	6.91 ± 0.82	1.75 ± 0.05
Fuc	0.24 ± 0.01	0.61 ± 0.01	0.19 ± 0.01	1.94 ± 0.01	1.06 ± 0.02	0.20 ± 0.01
GalA/Rha	7.56	2.68				

“—”: not detected.



### 3.4. FTIR spectra analysis

The infrared spectra of the six LMPs are presented in Fig. 2. The broadly stretched intense peak at  $3414\text{ cm}^{-1}$  is the characteristic absorption of hydroxyl groups, which was indicative of the strong inter- and intra-molecular interactions of the polysaccharide chains [24]. The weak absorption bands at about  $2927\text{ cm}^{-1}$  were attributed to C—H stretching vibrations of these LMPs [25]. The degree of methylation (DM) of pectic polysaccharides can be estimated by dividing the signal ascribed to carboxylic ester by the sum of the signal ascribed to carboxylic ester and carboxylic acid groups. The relatively strong absorption peak at  $1620\text{ cm}^{-1}$  was due to the stretching vibration of a free carboxylic carbonyl group and no obvious absorption attributed to carboxylic ester could be observed, suggesting the presence of non-esterified pectic polysaccharide. The absorption band at  $1409\text{ cm}^{-1}$  corroborates the presence of free carboxylic acids in all of the LMPs [7] and the uronic acid contents in both LMP1 and LMP2 were higher than other LMPs, which is consistent with the monosaccharide composition analysis. The signals at  $1029\text{ cm}^{-1}$  verified the presence of the glucose in the LMPs and the glucose was further demonstrated to be the main component in LMP6. In addition, the strong absorption band at  $1077\text{ cm}^{-1}$  was attributed to the arabinogalactans in the backbone, suggesting the presence of arabinogalactans in LMP2–LMP6 [26]. The absorption peaks around  $860\text{ cm}^{-1}$  and  $754\text{ cm}^{-1}$  are the characteristic peaks of  $\alpha$ -type glycosidic bonds, thus indicating that  $\alpha$ -type linkage was the main glycosidic linkage in all of the LMPs [7,27].

### 3.5. $^1\text{H}$ NMR analysis

The structural features of LMPs were further analysed by the  $^1\text{H}$  NMR spectra. As shown in Fig. 3, the anomeric proton signals in the relatively high field were present at 4.95–5.5 ppm, suggesting that the type of glycosidic bond was mainly  $\alpha$  glycosidic in all of the LMPs. The result was consistent with the FT-IR spectra analysis. Notably, no the protons of methyl ester groups or acetyl groups were detected, further indicating that polysaccharides from canning processing water were non-esterified. Chemical shifts in the  $^1\text{H}$  spectrum of  $\delta 5.39$  ppm were

attributed to the anomeric H-1 proton of  $\alpha\text{-D-GlcP}$  [28] and the signal intensity of LMPs in the NMR spectra was in accordance with the monosaccharide composition assay. Both LMP1 and LMP2 had a high proportion of GalA and showed well-resolved signals at 5.08 ppm. The AraF units usually attached as monomers or oligomers to the galactan chains and were detected by the anomeric proton at 5.3–5.05 ppm in LMP1–LMP5 [29]. The signals at 5.23 ppm and 4.67 ppm were assigned to the H-1 of RhaP and H-1 of Galp, respectively and the signals at around 1.15 ppm were assigned to the Rha residues. The data collected indicated great structural differences among the LMPs.

### 3.6. Top-down analysis of LMP1 and LMP2

In recent years, hydrophilic interaction chromatography (HILIC) coupled to mass spectrometry (MS) has proven to be a valuable technique for detailed separation and characterization of a wide range of pectic oligosaccharides [4]. However, manual analysis of the glycan profiling LC/MS data is extremely time-consuming. Therefore, GlycReSoft was used for efficient profiling of these LMPs. As the most degraded component, we conducted a top-down analysis on to clarify their structural properties. We excluded fractions with a relative abundance below 0.01 and the results are presented in Fig. 4. Notably, more oligosaccharide information was obtained for LMP2 than LMP1, indicating that LMP2 possessed a richer variety of oligosaccharides than LMP1. Specifically, the oligosaccharide components in LMP1 were mainly concentrated within 12 sugar residues and the content of the [6, 0, 0] fraction was the highest, which is consistent with the monosaccharide analysis. In comparison with LMP1, the information on oligosaccharide fractions of larger molecular weight was acquired, such as [6, 8, 6], [6, 10, 6], [8, 8, 6] and the degrees of polymerization (DP) of LMP2 ranged from 18 to 28. Interestingly, the oligosaccharide components in both LMP1 and LMP2 were almost the same oligosaccharides.

### 3.7. Cell viability assay

Considering that the biological activity of pectic polysaccharides depends on the oligosaccharide composition and DP distribution [30], the

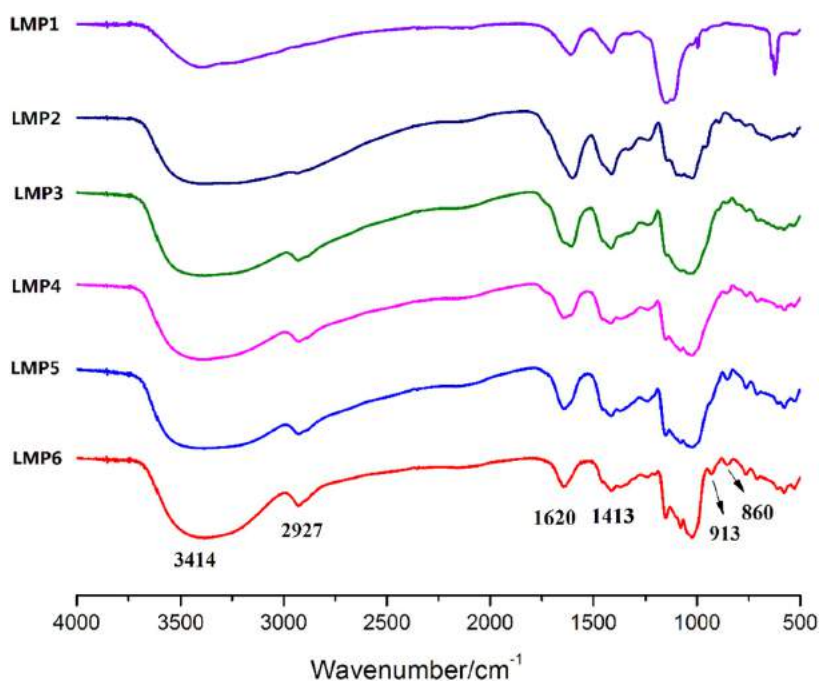
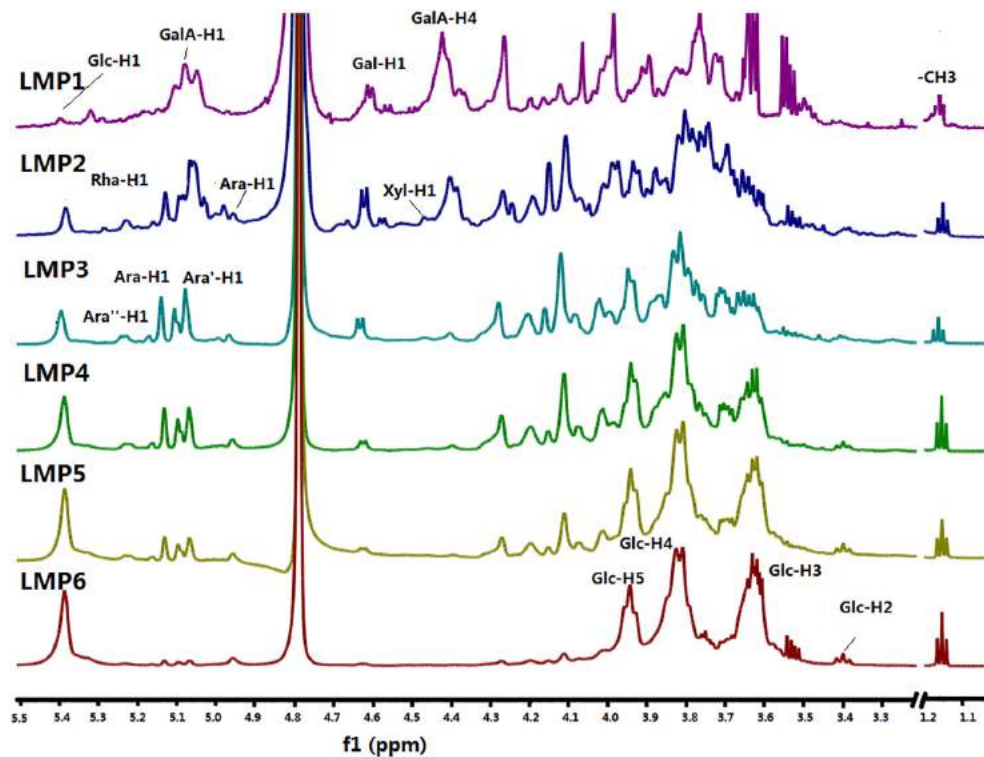
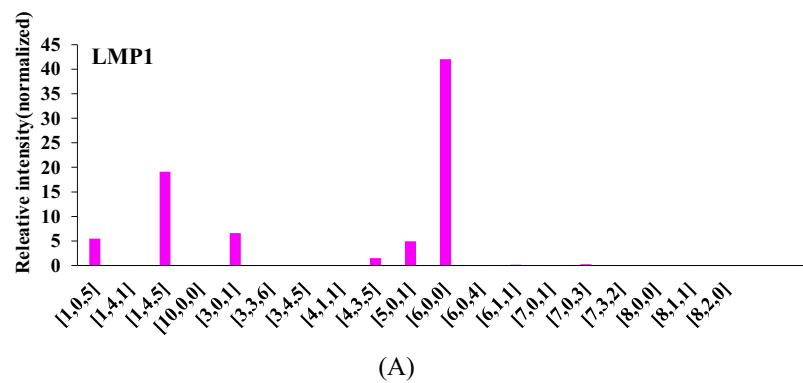
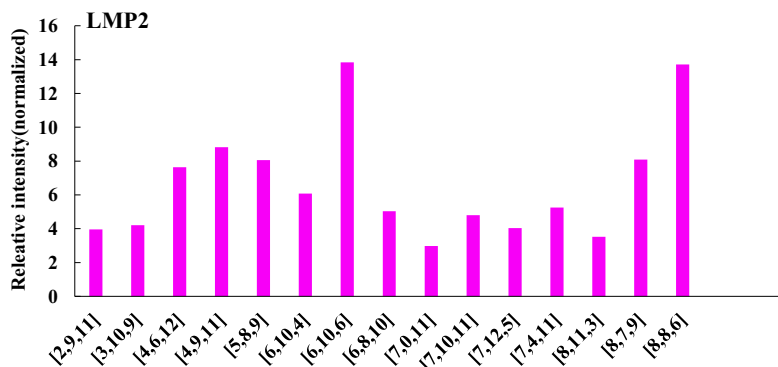


Fig. 2. The FT-IR spectra of LMPs.

Fig. 3. The  $^1\text{H}$  NMR spectra of LMPs.

(A)



(B)

Fig. 4. Top-down approach using HILIC LC-FT-ESI-MS to characterize LMP1 and LMP2. (A) Detected LMP1 oligosaccharides. (b) Detected LMP2 oligosaccharides. The oligosaccharides are labelled with the number of [HexA, Pentose, Hexose] moieties present in each structure.

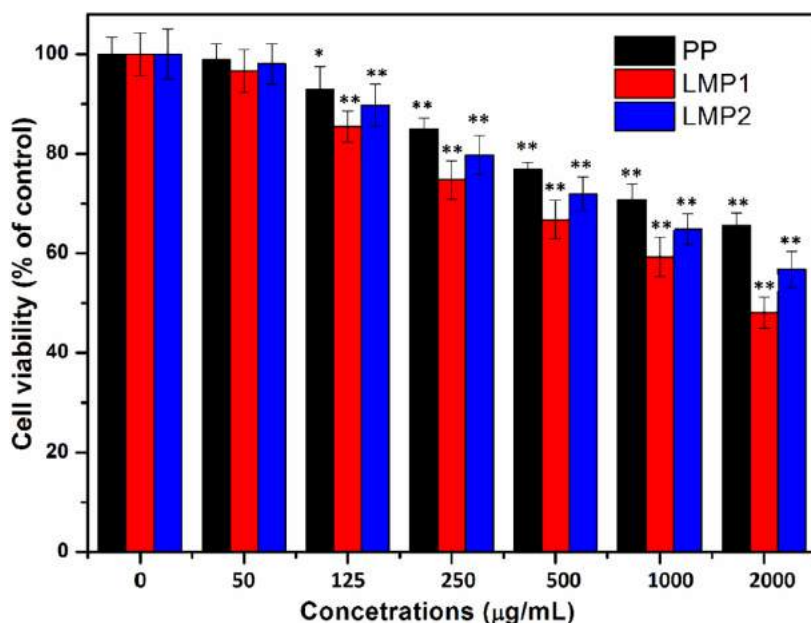


Fig. 5. Effects of native PPs, LMP1 and LMP2 on the proliferation of MCF-7 cells. Cells were cultured in the presence of PPs and LMP3 (0–2000 µg/mL) for 48 h and cell growth was determined by a MTT assay. Data are presented as mean  $\pm$  S.D. (\*)  $p < 0.05$  and (\*\*)  $p < 0.01$  indicate statistically significant differences versus blank control groups.

native pectic polysaccharides LMP1 and LMP2 were chosen to evaluate antiproliferative activity. As shown in Fig. 5, both LMP1 and LMP2 significantly inhibited the proliferation of MCF-7 cells in a dose-dependent manner. However, the native pectic polysaccharide showed much more moderate antiproliferative activity against MCF-7 cells. At 2000 µg/mL, LMP1 possessed the highest antiproliferative activity against MCF-7 cells with an inhibition rate of  $52 \pm 3.12\%$ , which is significantly higher than that of LMP2 at the same concentration ( $44.2 \pm 3.63\%$ ). Obviously, the order of the antiproliferation activity against MCF-7 of the two LMPs is positively correlated with the uronic acid content. Galectin 3, one of the  $\beta$  galactoside binding proteins that bind to the carbohydrate portion of cell surface glycoconjugates, is closely associated with their development and malignancy. Galactoside-containing molecules derived from pectic polysaccharides have been demonstrated to interact with a galectin 3-type lectin at the surface of proliferating mammalian cancer cells [31,32], thus preventing tumour growth. Both LMP1 and LMP2 are of small molecular size, so they can fit thoroughly on the galectin 3 molecule, particularly on the carbohydrate-binding domain (CBD) via its specific sugar  $\beta$  Gal residues. The basic amino acid constituents of CBD with enriched amounts of arginine may also strengthen the binding of LMP via its anionic nature due to the presence of an enriched amount of uronic acid. Therefore, the inhibitory effect of LMP1 on MCF-7 cells with 44.65% uronic acid content was higher than LMP2 with 19.48% uronic acid content. Despite the relatively high content of uronic acid and galactose in pectic polysaccharides, the low antiproliferation activity in both LMP1 and LMP2 indicated the importance of molecular size in polysaccharide binding to the galectin 3 of cancer cells [33]. Indeed, a relationship of low molecular weight pectic polysaccharides with higher uptake in cancer cells and stronger anticancer activity has been demonstrated [11].

#### 4. Conclusions

In the present study, an efficient Fenton system was developed for the fast preparation of pectic oligosaccharides. After purification by Superdex 30, six fractions, LMP1, LMP2, LMP3, LMP4, LMP5 and LMP6, were obtained. The monosaccharide analysis indicated that GalA was the principle component of both LMP1 and LMP2 and Ara and Gal

were the major neutral saccharides, while LMP3, LMP4, LMP5 and LMP6 were mainly composed of glucose at the ratios of 33.28, 61.75, 69.33 and 82.28%, respectively. FT-IR and NMR spectra showed that all of the LMPs were non-esterified polysaccharides and the type of glycosidic bond was mainly  $\alpha$  glycosidic. A top-down analysis was further applied to determine the composition and preliminary structural features of LMP1 and LMP2. The *in vitro* antitumor activity of pectic oligosaccharides including LMP1 and LMP2 was revealed via MTT assays. The results showed that both LMP1 and LMP2 showed significant antitumor activity against MCF-7 cells while pectic polysaccharide had moderate activity, which could be due to their structural characteristics, including molecular weight, uronic acid content and monosaccharide composition. These results suggested that pectic oligosaccharides prepared from citrus canning process water is suitable for use as functional foods and are potential therapeutic agents for human cancer. However, the lack of systematic studies about their structures and acting mechanisms does not allow us to understand the structure-function relationship. Future research must be performed on these subjects to confirm their physiological properties as well as the production of these compounds on a larger scale.

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