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Structural characterization and antitumor activity of a pectic polysaccharide from *Codonopsis pilosula*

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ABSTRACT

A pectic polysaccharide (CPP1b) was at first isolated from *Codonopsis pilosula*. Sugar analysis revealed that CPP1b is composed of rhamnose (Rha), arabinose (Ara), galactose (Gal) and galacturonic acid (GalA) with a molar ratio of 0.25:0.12:0.13:2.51. The result of esterification assay showed that about 46.7 ± 0.4% of carboxylic groups in GalA residues existed as methyl ester. Combined with chemical and spectroscopic analyses, a preliminary structure of CPP1b was proposed as follows: 1,4-linked α-D-Gal*p*A and 1,4-linked α-D-Gal*p*A6Me interspersed with rare 1,2-linked β-L-Rha*p*, 1,2,6-linked α-D-Gal*p* and terminal α-L-Ara*p*. CPP1b had an average molar mass and root-mean square radius (RMS) of 1.45×10^5 Da and 29.7 nm, respectively, and presented a linear random coil conformation in 0.9% NaCl. The ultrastructure of CPP1b was further investigated by transmission electron microscope (TEM) and scanning electron microscope (SEM). CPP1b exhibited obvious cytotoxicity to human lung adenocarcinoma A549 cells in a dose- and time-dependent manner.

1. Introduction

The roots of *Codonopsis pilosula* (Franch.) Nannf. (Campanulaceae) (CP), a well-known traditional Chinese herbal medicine, has long been prescribed in traditional folk medicine in China, Japan and Korea. The main constituents of CP include polysaccharides, saponins, sesquiterpenes, polyphenolic glycosides, alkaloids, polyacetylenes, essential oils, and phytosteroids (Heidrun & Heidrun, 1994; Mizutani et al., 1988; Wang, Ma, Tu, & Xu, 1995; Wang & Wang, 1996). A number of new components of CP have been found in recent years, e.g., phenylpropanoid derivatives and triterpenyl esters (Song, Chou, Zhong, & Wang, 2008; Wakana, Kawahara, & Goda, 2011). Modern pharmacology research has shown that CP has antitumor, antimicrobial, and antioxidant functions (Liu, Cai, & Shao, 1988; Luo et al., 2007; Shan, Yoshida, Sugiura, & Yamashita, 1999).

As the main ingredient of water soluble CP extracts, the CP polysaccharide is considered one of the most important substances responsible for the therapeutic function of CP. In previous studies, the significant antitumor and immunoenhancing activities of CP were attributed to the effect of the polysaccharide (Zong, Cao, & Wang, 2012). Xin et al. reported a CP polysaccharide (CPPA) that could not only induce a potent inhibitory effect on the invasion and migration potential of human epithelial ovarian cancer HO-8910 cells in vitro but also had an efficient anti-proliferation effect on tumor cells (Xin et al., 2012). Sun and his colleagues obtained a water soluble CP polysaccharide that was determined to be a safe, efficacious adjuvant for use in vaccines against both pathogens and cancer (Sun, 2009). It was confirmed that the side chains attached to the backbone of the CP polysaccharide (CPPW1) played a pivotal role in fighting the tumor and activating the immune system (Xu, Liu, Yuan, & Guan, 2012). Recently, great interest has been focused on finding reliable methods to study the structure and the structure-activity relationships of polysaccharides. Unfortunately, most current reports on CP polysaccharides focused on only its pharmacological activity and therapeutic effects. Only a few polysaccharides from CP have been characterized. Many water soluble polysaccharides have been isolated from CP, and the structure of a water soluble CP polysaccharide (CPPS3) was reported by Zhang et al. (Zhang et al., 2005; Zhang, Zhang, Yang, & Liang, 2010). Sun and Liu investigated the basic structure of a neutral CP polysaccharide, which was composed of β-D-galactopyranosyl, α-L-arabinose and α-D-rhamnopyranosyl, with a molecular weight of 1.1×10^4 Da (Sun & Liu, 2008).

Pectins are an important family of heterogeneous polysaccharides from the plant cell wall (Albersheim, Darvill, O'Neill, Schols, &

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Voragen, 1996). The complex structures of pectins consist of homogalacturonan (HG) and rhamnogalacturonans (RG-I and RG-II). HG segments or 'smooth regions' have linear chains of (1→4)-linked a-D-galacturonic acid (GalA) residues which can be partly methyl esterified at COOH-6, while the hydroxyl groups at position O-2 or O-3 can be acetylated (Pilnik & Voragen, 1970; Rolin, 1993). HG sections might be interspersed by rhamnogalacturonan elements. The RG-I contains galactose, arabinose and arabinogalactose side chains of different length, whereas, RG-II contains various rare sugars and complex oligosaccharide chains (Brett & Waldron, 1996). It has been reported that ginseng contained pectins which show significant biological activities, such as the immunomodulating, anticancer, antioxidant, hypoglycemic activities and antiadhesion against pathogenic bacteria et al. (Gao, Hiyohara, Cyong, & Yamada, 1989; Konno, Sugiyama, Kano, Takahashi, & Hikino, 1984; Lee et al., 2006; Tomoda et al., 1993). For a long time, CP was used as a cheap substitute for ginseng in China (Wang, Ng, Yeung, & Xu, 1996). However, no study on the pectin from CP has yet been published until now.

Our present work isolated and characterized a pectic polysaccharide from CP, designated CPP1b, and investigated its cytotoxicity on human lung adenocarcinoma A549 cells firstly.

2. Materials and methods

2.1. Plant material and chemicals

Dried CP was collected in Wen County (Gansu Province, China). A voucher specimen was identified by Prof. Yinsuo Zhou, School of Pharmacy, Lanzhou University. Standard monosaccharides (including arabinose, rhamnose, xylose, galactose, glucose, mannose, glucuronic acid and galacturonic acid) were purchased from the National Institutes for Food and Drug Control (Beijing, China). DEAE-Cellulose was purchased from Whatman Co. (Maidstone, UK). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Amresco Co. (USA). Methotrexate (MTX) was purchased from the National Institutes for Food and Drug Control (Beijing, China). Trifluoroacetic acid (TFA), dimethyl sulfoxide (DMSO), sodium borohydride ($NaBH_4$), methyl iodide (CH_3I) and other reagents were all of analytical grade.

2.2. General analysis

Total carbohydrate contents were determined by the phenol-sulfuric acid colorimetric method using glucose as a reference (Dubois, Gilles, Hamiltion, Rebers, & Smith, 1956). The protein content was determined by Lowry's method with bovine serum albumin (BSA) as a standard (Bensadoun & Weinstein, 1976). Ultraviolet and visible (UV–vis) absorption spectroscopy were recorded with a UV spectrophotometer (UV-1700, Shimadzu, Japan).

2.3. Extraction, purification and fractionation of polysaccharides

The extraction of the CP polysaccharides was performed according to the method reported by Sun, Liu, and Kennedy (2010) with a slight modification. Briefly, after being defatted with ethanol (95%), dried crude CP powder (1000 g) was extracted with 9 L of water at 90 °C for 2 h, repeated 3 times. The solutions were combined, centrifuged (4000 rpm, 10 min) and concentrated to 1/4 the volume of the original solution under reduced pressure at 60 °C. The concentrated aqueous solution was submitted to graded precipitation with 0.5, 1, 3, and 4 volumes of ethanol in sequence. The four precipitate fractions (CPP1, CPP2, CPP3 and CPP4) were collected by centrifugation, individually washed successively with ethanol and acetone, and then lyophilized. The antioxidation of the four fractions against DPPH and hydroxyl radical was studied before this experiment (data not shown). The CPP1 presented stronger antioxidation activity among the four fractions, which inspired our further study interest to CPP1.

The process of purification and fractionation of CPP1 was is described below, following Scheme 1. CPP1 was deproteinated according to the Sevag method (Sevag, 1934). Briefly, 15 mg proteinase was added to the saturated CPP1 solution (enzyme:protein = 1:50), dialyzed in distilled water at 37 °C for 24 h (changing water every 4 h), and then the sugar solution was treated with the Sevag reagent (1-butanol:chloroform = 1:4) and fully oscillated and centrifuged. The process was repeated until the supernatant contained no free protein as assessed by Lowry's method. After decoloration by H_2O_2, the supernatant was dialyzed with running water and distilled water using a dialysis membrane with a molecular weight cut off of 3500 Da for 24 h.

An aliquot (100 mg) of CPP1 was further purified by anion-exchange chromatography on a column (2.6 × 70 cm, i.d.) packed with DEAE-cellulose filtration medium. The column was eluted with distilled water and a NaCl gradient (0.1–1 M) in sequence, and fractions were collected at a flow rate of 0.5 mL/min (each test tube contains 5 mL) and monitored by the phenol-sulfuric acid assay (Dubois et al., 1956). The acidic polysaccharide was obtained in the fraction eluted with 0.2 M NaCl. After dialysis and freeze-drying, the acidic polysaccharide, named CPP1b, was obtained. This sample was used for structural characterization and investigation of antitumor activity.

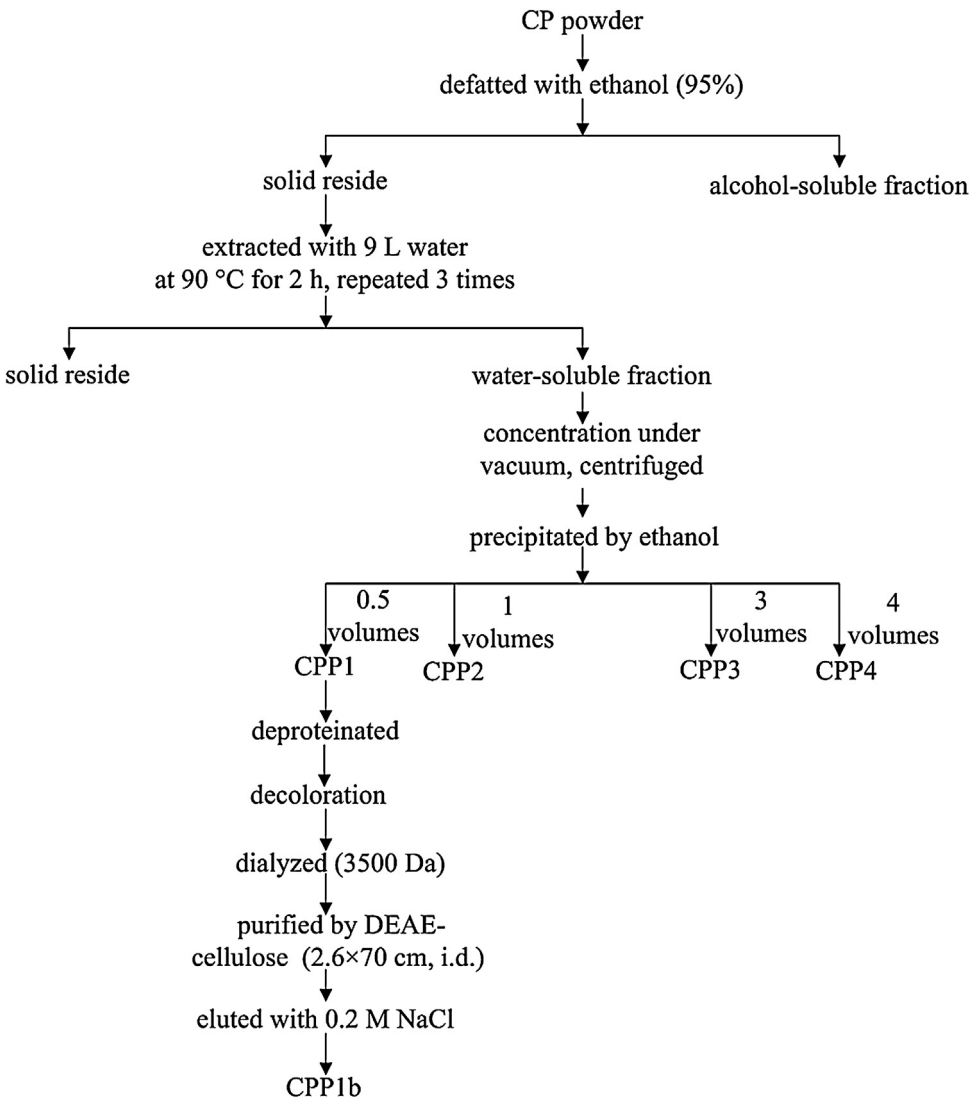
2.4. Sugar composition

CPP1b (10 mg) was hydrolyzed with 2 M TFA (2 mL) for 3 h at 120 °C to release the component monosaccharides. The hydrolyzed monosaccharides (with inositol as the internal control) were derivatized by the aldononitrile acetate method (Mawhinney, Feather, Barbero, & Martinez, 1980) and analyzed by gas chromatography (GC, Shimadzu, Japan). The quantitative analysis was performed with a capillary column (50 m × 0.25 mm i.d.) of OV-101, programmed at 15 °C/min from 175 °C to 190 °C and held at the latter temperature for 5 min, followed by an increase of 5 °C/min to 250 °C and held there for 1.5 min. The combined standards, including rhamnose, arabinose, xylose, mannose, glucose, galactose, were treated using same method.

The uronic acid contents of CPP1b were determined using the method described by Zhang (1994). The treated samples were also analyzed by GC. The quantitative analysis was performed using a capillary column (50 m × 0.25 mm i.d.) of OV-101, held at 160 °C for 4 min, then programmed at 5 °C/min to 190 °C and held at this temperature for 4 min, followed by an increase of 3 °C/min to 210 °C and held at this temperature for 15 min, and finally increased at 10 °C/min to 260 °C and held for 5 min.

2.5. Determination of esterification

Internal calibration curves for quantification of methanol and acetic acid were prepared by adding the standards in the concentration range between 20 and 100 μg/mL for methanol and acetic acid, and isopropanol as the internal standard. The saponification of CPP1b occurred by treatment with 0.8 mL of 2 M NaOH at 25 °C. After 1 h, the reaction was terminated by the addition of 0.8 mL of 2 M HCl, and pH was adjusted to 2.0, as described (Wang, Wang, Shi, Duan, & Wang, 2012). The GC oven temperature program used for methanol was: stay at 65 °C (held for 8 min) and program used for acetic acid was: stay at 100 °C (held for 8 min). The flow rate of the carrier gas (N2) was set at 2 mL/min.



Scheme 1. Scheme for extraction, purification and fractionation of CPP1b.

2.6. Homogeneity and molecular properties

The homogeneity of CPP1b was checked by high-performance gel permeation chromatography (HPGPC), which was conducted by a high performance liquid chromatography (HPLC) system, equipped with a 600E pump (Waters 600) and differential refractometer detector (Waters 2414). Two columns were used in series, with Ultrahydrogel™ 1000 and Ultrahydrogel 500 (Waters). The eluent was ultrapure water at 0.8 mL/min. The sample, previously filtered through a membrane (0.45 μm, Millipore), was injected (10 μL) at a concentration of 2 mg/mL.

HPGPC coupled with multi-angle laser light scattering (MALLS, Wyatt DAWN-EOS) was applied to determine the molecular weight and morphology of CPP1b. The mobile phase was composed of 0.9% NaCl + 0.2% NaN_3, and passed through the HPLC column at a flow rate of 0.8 mL/min. The CPP1b sample at a concentration of 10 mg/mL was loaded into the chromatography system (30 μL). Astra software was used to analyze the selected light scattering peak using Zimm plots to generate the molecular parameters including the molar mass (Mw), the root-mean square (RMS) radius and polydispersity index (Mw/Mn) of the polymer molecules.

2.7. Methylation analysis

Uronic acids were reduced to neutral polysaccharides before methylation following the method of Singthong, Cui, Ningsanond, and Goff (2004). Briefly, the free uronic acids were activated with a carbodiimide (1-cyclohexyl-3-(2-morphol-inoethyl)-carbodiimide methyl-*p*-toluenesulfonate, Sigma) and reduced with $NaBH_4$. The methylation analysis was carried out according to the method of Needs and Selvendran (1993). The dried methylated sample was hydrolyzed and isolated as partially methylated alditol acetates (PMAA), as described by Harris, Henry, Blakeeny, and Stone (1984). The PMAA were analyzed by gas chromatography–mass spectrometry (GC–MS, Varian-450-GC-320-MS, USA). The capillary column used was a DB-5 capillary column (50 m × 0.25 mm i.d., 0.25 μm film thickness) programmed at 5 °C/min from 150 °C to 170 °C, then 8 °C/min to 225 °C and held at this constant temperature for 1 min, and finally at 8 °C/min to 250 °C and held at this constant temperature for 5 min. He (helium) was used as the carrier gas. The temperatures of the interface and ion source were 250 °C and 200 °C, respectively.

2.8. Nuclear magnetic resonance (NMR) spectroscopy

CPP1b was dissolved in D_2O at a concentration of approximately 30 mg/mL, deuterium-exchanged 3 times by freeze-drying in D_2O. Both ^1H and ^{13}C NMR spectra were recorded on a 600 MHz Bruker INOVA 600NB NMR spectrometer (Brucker, Rheinstetten, Germany). The relaxation delays for the NMR experiment were 1.0 s. The double quantum filtered correlated spectrosopy (DQF-COSY), nuclear Overhauser effect spectroscopy (NOESY), heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple-bond correlation (HMBC) experiments were conducted at 60 °C.

2.9. Ultrastructure of CPP1b

For transmission electron microscopy (TEM), CPP1b was dissolved (5 mg/mL) in ultra-pure water and 10 μL was transferred to a Formvar film-coated copper grid. The solution was dried and negatively stained with phosphotungstic acid. After drying, the sample was observed in a JEOL JSM-6380 LV, operated at 80 kV. For scanning electron microscopy (SEM), the CPP1b powder was directly deposited on a carbon tape-coated aluminum stub and spattered with gold (Balzers/Union FL-9496) for 1 min and assayed in a JEOL JEM-1230, operated at 30 kV.

2.10. Antitumor assays

Human lung adenocarcinoma A549 cells were obtained from the School of Medicine at Lanzhou University (Lanzhou, China). The growth inhibitory rates of CPP1b to A549 cells were evaluated in vitro by MTT assay (Mosmann, 1983). A549 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), (Hsu, Kuo, & Lin, 2004; Tomatsu, Ohnishi-Kameyama, & Shibamoto, 2003) in a humidified 5% CO_2 atmosphere at 37 °C. Briefly, cells were seeded (5×10^3 cells/well) in medium (40 μL) in 96-well plates, respectively. After 24 h incubation, the CPP1b (0–400 μg/mL) which was dissolved in a medium was added to each well and incubated for 24 and 48 h at 37 °C in a CO_2 atmosphere. The experiment was conducted according with the following dosages: 0, 50, 100, 200, 400 μg/mL CPP1b without MTX, 200 μg/mL CPP1b with 8 μg/mL MTX. MTX was used as the positive control. All assays were done in quintuplicate. MTT (5 mg/mL; 10 μL) was added 48 h and 72 h later. After incubating the cells at 37 °C for 4 h, the supernatants were aspirated, and DMSO (100 μL) was added to each well. The absorbance was detected at 570 nm with a 96-well microplate reader. (Mode 680, Bio-Rad, Tokyo, Japan). The growth inhibitory rate to the tumor cells was determined as follows:

$$\text{Inhibition rate}(\%) = \left(1 - \frac{\text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}}\right) \times 100\%$$

2.11. Statistical analyses

The data were presented as means ± SD of five determinations. Statistical analyses were performed using one way analysis of variance (ANOVA, $p < 0.05$). Multiple comparisons of means were done by the least significance difference test. Significant difference between two groups was defined as $p < 0.05$. All computations were done by employing the statistical software (SAS, version 8.0).

3. Results

3.1. Properties and homogeneity

The crude polysaccharide, named CPP1, obtained from water extraction, accounts for 9.7% of the CP dried weight. After fractionation on DEAE-cellulose chromatography, CPP1b was obtained from the 0.2 M NaCl eluate and detected by the phenol-sulfuric acid colorimetric method with the total sugar content of 98.08%. CPP1b was free of protein as assessed by Lowry's method. CPP1b showed a single and symmetrical sharp peak on the HPGPC chromatogram, indicating that it is homogeneous. The average molar mass (Mw), the root-mean square (RMS) radius and polydispersity index (Mw/Mn) of CPP1b in 0.9% NaCl aqueous solution were 1.45×10^5 Da, 29.7 nm and 1.19, respectively. The conformation plot based on the molar mass versus RMS radius elution volume displayed an irregular shape with a gradient of 0.45, which indicated that CPP1b was a linear random coil in 0.9% NaCl aqueous solution (Thielking & Kulicke, 1998).

3.2. Sugar composition of CPP1b and determination of esterification

GC analysis revealed that CPP1b was composed of rhamnose (Rha), arabinose (Ara), galactose (Gal) and galacturonic acid (GalA) at a molar ratio of 0.25:0.12:0.13:2.51 (nearly 2:1:1:20). The considerable proportion of uronic acid in CPP1b would make it difficult to perform the methylation experiment (Cui, 2005), so the carboxyl group of CPP1b had to be reduced before methylation analysis.

As determined, about 46.7 ± 0.4% of carboxylic groups in GalA residues of CPP1b existed as methyl ester.

3.3. Methylation analysis

The individual peaks of the PMAA from the GC–MS analysis were identified by their retention time and by comparison with the mass spectrum. The molar ratio of each sugar residue was calculated from the peak area in the GC chromatogram. The results showed the presences of four types of linkages 1,2-linked Rha*p*, terminal Ara, 1,2,6-linked Gal*p* and 1,4-linked Gal*p* at a molar ratio of 0.21:0.08:0.10:1.95 (nearly 2:1:1:20). As CPP1b was treated with $NaBH_4$ before methylation, 1,4-linked Gal*p* corresponds to GalA*p* as is evident from the data presented below. These data indicated that the main chain of CPP1b is composed of 1,4-linked GalA*p*, interspersed with 1,2-linked Rha*p*, 1,2,6-linked Gal*p* and terminal Ara.

3.4. 1D and 2D NMR analysis

Signals of CPP1b in 1D ^1H and ^{13}C NMR and 2D NMR (DQF-COSY, HMQC, HMBC, and NOESY) spectra were assigned as completely as possible, based on monosaccharide analysis, linkage analysis and chemical shifts reported in the literature. The ^1H NMR spectrum (Fig. 1a) shows six peaks in the anomeric region, which were arbitrarily labeled A, B, C, D, E and F. The intense anomeric proton signals δ 4.92 and 4.89 ppm corresponded to the methyl esterified Gal*p*A and non-methyl esterified α-D-Gal*p*A, respectively. The anomeric proton signals δ 4.79 and 4.74 ppm corresponded to H-1 of Rha*p*. The intense ^1H signal at 3.63 ppm (Fig. 1a), the ^{13}C signal at δ 55.4 ppm (Fig. 1b) and cross peak δ 3.63/55.4 and 4.98/72.5 ppm in the HMQC spectrum (Fig. 2b) coupled with the cross peaks δ 3.63/173 ppm in the HMBC spectrum (Fig. 3a) indicate that GalA is partly methyl esterified (Zhao et al., 2006). In the downfield region of ^{13}C NMR spectrum (Fig. 1b), signals at δ 173.4 and 177.7 ppm were assigned to the carboxyl groups of methyl esterified GalA and non-methyl esterified GalA, respectively. The weak resonances at

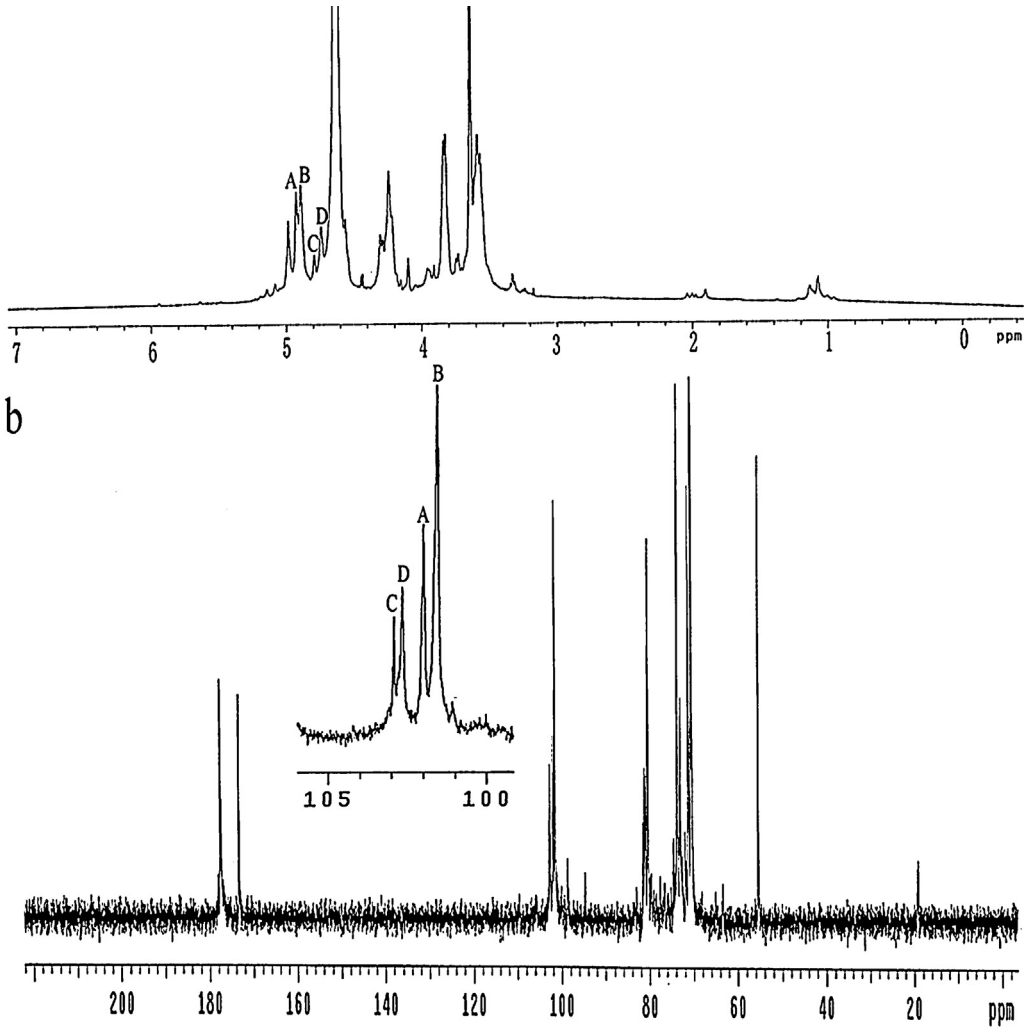


Fig. 1. ^{1}H (a) and ^{13}C (b) NMR of CPP1b isolated from *Codonopsis pilosula*.

δ 1.08 and 1.13 ppm correspond to the methyl group of Rha; the signals at δ 19.1 and 19.3 ppm also belong to the methyl group of Rha (Fig. 1b), which was confirmed by cross peaks δ 1.08/19.3 and 1.13/19.1 ppm in the high-field of the HMQC spectrum (Fig. 2b), corresponding to H-6/C-6 of Rha (Leone, Molinaro, Dubery, Lanzetta, & Parrilli, 2007). In addition, the weak signals about δ 2.00 ppm suggested that CPP1b contained a very small amount of O-acetyl groups.

In the case of residue A, the cross-peaks δ 4.92/3.56, 3.56/3.82, 3.82/4.24 ppm were detected, in the DQF-COSY spectrum (Fig. 2a). Because δ 4.92 ppm corresponded to H-1 of residue A, δ 3.56, 3.82 and 4.24 ppm corresponded to H-2, H-3 and H-4 of residue A. The small *J*-coupling constants of $^{3}J_{\text{H-1, H-2}} \sim 3$ suggested that the residue is α-linked (Takeda, Nakamori, Hatta, Yamada, & Koizumi, 2003). Based on the ^{1}H NMR assignment from DQF-COSY spectra, the carbon signals in the HMQC spectrum for residues A was easily assigned (Fig. 2b). The cross-peaks δ 4.92/101.6, 3.56/72.0, 3.82/73.2 and 4.24/81.2 corresponded to H-1/C-1, H-2/C-2, H-3/C-3 and H-4/C-4 of residue A. The cross peaks δ 3.63/55.4 and 4.98/72.5 ppm in the HMQC spectrum (Fig. 2b) coupled with the cross peak δ 4.98/173.4 and 3.63/173.4 ppm in the HMBC spectrum (Fig. 3a) indicate that residue A is methyl esterified (Zhao et al., 2006). As δ 173.4 ppm corresponded to C-6 of residue A, δ 4.98/72.5 ppm corresponded to H-5/C-5 of residue A, δ 3.63/55.4 ppm corresponded to methoxyl group. When compared to chemical shifts of standard Gal*p*A (data not shown), signal C-4 δ 81.2 ppm indicated that residue A is 4-linked α-D-Gal*p*A6Me residue, which corresponded to the result of the methylation analysis. The complete ^{1}H and ^{13}C NMR signal assignments for residue A are summarized in Table 1.

As for residue B, the cross-peaks δ 4.89/3.59, 3.59/3.83, 3.83/4.30 ppm were detected, in the DQF-COSY spectrum (Fig. 2a). Because δ 4.89 ppm corresponded to H-1 of residue B, δ 3.59, 3.83 and 4.30 ppm corresponded to H-2, H-3 and H-4 of residue B. The small *J*-coupling constants of $^{3}J_{\text{H-1, H-2}} \sim 3$ suggested that residue B is α-linked (Takeda et al., 2003). In the HMQC spectrum, the cross-peaks δ 4.89/101.1, 3.59/71.2, 3.83/73.1 and 4.30/80.4 ppm were detected. Because δ 4.89, 3.59, 3.83, 4.30 corresponded to H-1, H-2, H-3 and H-4 of residue B, δ 101.1, 71.2, 73.1 and 80.4 ppm corresponded to C-1, C-2, C-3 and C-4 of residue B. In downfield region of the HMBC spectrum (Fig. 3a), the cross-peak δ 4.69/177.7 ppm was detected. Because δ 177.7 ppm corresponded to C-6 of residue B, δ 4.69 ppm corresponded to H-5 of residue B. when compared to chemical shifts of standard Gal*p*A (data not shown), signal δ 80.4 ppm of C-4 indicated that residue B is 4-linked α-D-Gal*p*A residue, which corresponded to the result of the methylation analysis. The complete ^{1}H and ^{13}C NMR signal assignments for residue B are summarized in Table 1.

In the case of residue C and D, the cross-peaks δ 1.08/3.59 and 1.13/3.60 ppm, δ 3.59/3.37 and 3.60/3.38 ppm, δ 3.37/4.43 and 3.38/4.44 ppm were detected, in the DQF-COSY spectrum (Fig. 2a). Because the peaks δ 1.08 and 1.13 ppm corresponded to H-6 of Rha*p*,

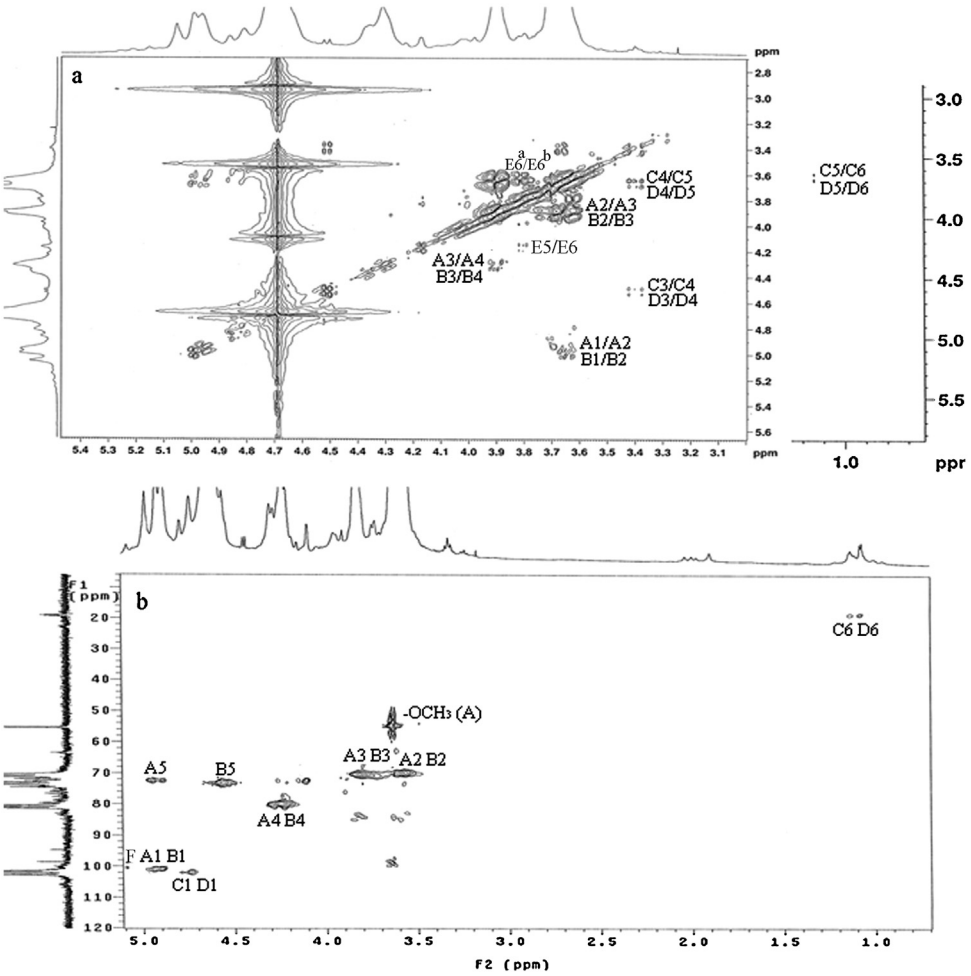


Fig. 2. (a) DQF-COSY spectrum of CPP1b, (b) HMQC spectrum of CPP1b isolated from *Codonopsis pilosula*.

δ 3.59 and 3.60 ppm, δ 3.37 and 3.38 ppm, δ 4.43 and 4.44 ppm corresponded to H-5, H-4 and H-3 of Rha*p*. The signals δ 4.79 and 4.74 ppm are attributed to β-L-Rha*p*, which were the only signal in the anomeric region that remained unassigned after the assignment of all other anomeric signals because the chemical shifts of its anomeric protons is lesser than 5.0 ppm (Glushka et al., 2003). Complete carbon signals were obtained from the HMQC spectrum (Fig. 2b). The complete ^{1}H and ^{13}C NMR signal assignments for residue C and D are summarized in Table 1.

The less signals of residue E and F was caused by less content of Gal and Ara. In the DQF-COSY spectrum (Fig. 2a), The signals δ 3.73/3.51 and 3.73/4.15 ppm corresponded to H^{a}-6/H^{b}-6 and H-6/H-5 of Gal*p*. In the ^{1}C NMR spectrum (Fig. 1b), the signal δ 63.4 ppm corresponded to C-5 of Ara. In the anomeric region of HMQC spectrum, the signal δ 5.08/100.0 corresponded to H-1/C-1 of Ara. The complete ^{1}H and ^{13}C NMR signal assignments for residue E and F are summarized in Table 1.

The sequence of the residues in the repeating unit was determined based on the cross-peaks observed in the NOESY (Fig. 3b) and HMBC spectra (Fig. 3a). The cross peak δ 4.89/4.24 ppm was detected in the NOESY spectrum (Fig. 3b). Because δ 4.89 ppm corresponded to H-1 of residue B, and δ 4.24 ppm to H-4 of residue A, it was concluded that residues B and A are connected to each other by a 1, 4-O glycosidic bond. The cross peak δ 4.92/4.30 ppm was detected in the NOESY spectrum (Fig. 3b). Because δ 4.92 ppm corresponded to H-1 of residue A, and δ 4.30 ppm to H-4 of residue

Table 1
^{13}C NMR and ^{1}H NMR chemical shifts (δ in ppm) for CPP1b.

Sugar residues	C-1/H-1	C-2/H-2	C-3/H-3	C-4/H-4	C-5/H-5	C-6/H-6	—OCH_3
→4)-α-D-Gal*p*A6Me-(1→	101.6	72.0	73.2	81.0	72.5	173.4	55.4
(A)	4.92	3.56	3.82	4.24	4.98		3.63
→4)-α-D-Gal*p*A-(1→	101.1	71.2	73.1	80.4	73.7	177.7	
(B)	4.89	3.59	3.83	4.30	4.69		
→2)-β-L-Rha*p* ^{I}-(1→	102.9	81.2	–	71.3	73.2	19.3	
(C)	4.79	4.23	4.43	3.37	3.59	1.08	
→2)-β-L-Rha*p* ^{II}-(1→	102.7	81.5	–	71.2	73.7	19.1	
(D)	4.74	4.21	4.44	3.38	3.60	1.13	
→2,6)-α-D-Gal*p*-(1→	–	77.6	–	–	73.7	71.2	
(E)	5.14	4.18	–	–	4.15	3.51, 3.73	
→1)-α-L-Ara*p*-(1→	101.1	70.6	70.5	–	63.4		
(F)	5.09	–	3.32	4.42	3.30		

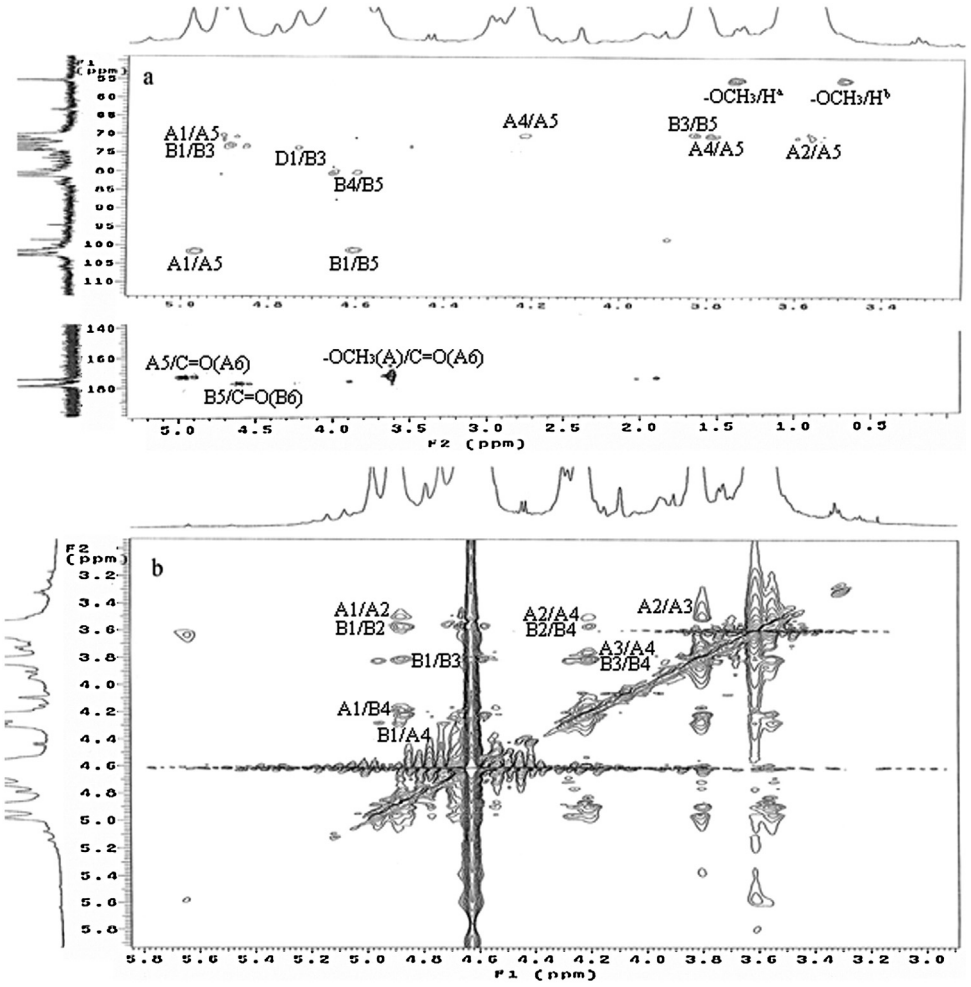


Fig. 3. (a) HMBC spectrum of CPP1b, (b) NOESY spectrum of CPP1b isolated from *Codonopsis pilosula*.

B, it was concluded that residues A and B are connected to each other by a 1, 4-O glycosidic bond. The cross-peaks δ 3.59/4.89, 3.83/4.89, 3.83/4.30 and 3.56/4.30 were observed in the NOESY spectrum (Fig. 3b), which were assigned to A2/A1, A3/A1, A3/A4 and B2/A4, respectively. The cross peak δ 4.74/73.1 ppm was detected in the HMBC spectrum (Fig. 3a). Because δ 4.74 ppm corresponded to H-1 of residue D, and δ 73.1 ppm to C-3 of residue B, it was concluded that minor residues B are connected to residues D by a 1, 4-O glycosidic bond. The cross-peaks δ 3.74/55.4 and 3.51/55.4 ppm were observed in the NOESY spectrum (Fig. 4b), and δ 3.63 ppm located in the middle of δ 3.74 and 3.51 ppm, which were assigned to methoxyl group of residue A.

According to the classification of pectic polysaccharides as well as the aforementioned results, CPP1b was supposed as polygalacturonan interspersed with rhamnogalacturonan in the main chain, which could be classified as RG-I contained Ara side chains (Brett & Waldron, 1996). Based on the experimental data presented above, the residue sequence of the repeating unit shown is Fig. 4 is proposed.

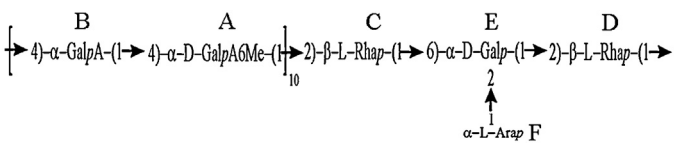


Fig. 4. Structure of CPP1b, GalpA stands for galacturonic acid, GalpA6Me stands for methyl esterified galacturonic acid.

3.5. Ultrastructure

Fig. 5 shows the typical micrographs of CPP1b observed by TEM (Fig. 5a and b) and SEM (Fig. 5c and d). As the specimens were prepared using different routes depending on the microscopy method used, each technique may have induced various artifacts. TEM observations revealed that the ultrastructure of CPP1b is mainly composed of randomly distributed ovoid-shape particles, with diameters of approximately 100 nm. SEM showed a surface with a sheet-like appearance.

3.6. Antitumor activities

We investigated the cellular cytotoxicity of CPP1b at different concentrations (0, 50, 100, 200, 400 μg/mL) without MTX and with 200 μg/mL with MTX on human lung cancer A549 cells. As shown in Fig. 6, cytotoxicity against A549 cells was found for CPP1b at different concentrations in a dose- and time-dependent manner. After 24 h coculture with A549 cells, the growth inhibition rates on A549 cells increased from 46.6% to 61.9%, at CPP1b concentrations of 50–400 μg/mL, respectively. After 48 h, the inhibition rates increased from 46.8% to 65.4% at the same concentrations. It is worth noting that CPP1b can significantly improve the antitumor activity of MTX after 48 h coculture with A549 cells as compared to

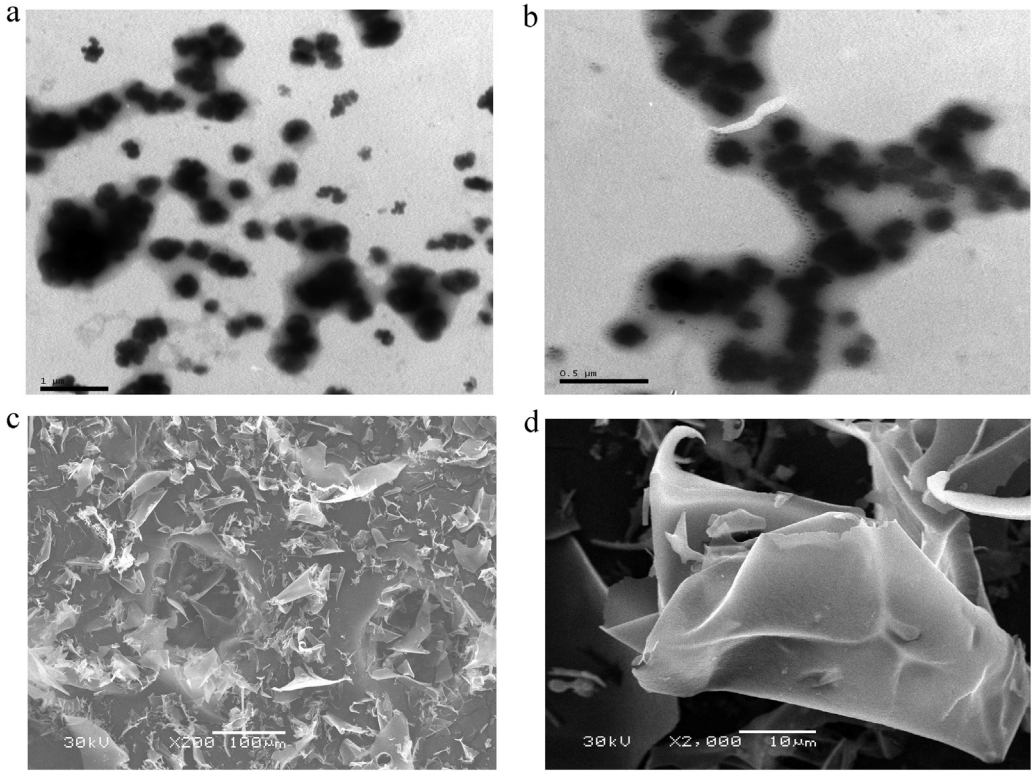


Fig. 5. Electron microscopy of CPP1b. (a) TEM of CPP1b presenting amorphous particles, scalebar is 1 μm. (b) Low magnification image of the amorphous particles, scalebar is 0.5 μm. (c) SEM of CPP1b revealing a surface with a sheet appearance, scalebar is 100 μm. (d) Detailed view of a sheet appearance, scalebar is 10 μm.

the positive control ($p < 0.01$). The enhanced cytotoxicity shown by CPP1b indicates that it may exhibit synergistic activity with MTX against A549 cells. These results revealed that CPP1b could become a potential antitumor or adjuvant antitumor drug.

4. Discussion

The antitumor properties of polysaccharides are primarily mediated through the following three approaches: direct cytotoxicity, immunoenhancement, and synergistic effects in combination treatment with conventional antitumor drugs. Direct cytotoxicity involves the interference of polysaccharides with cancer induction, growth, and progression by inducing cellular apoptosis and cell cycle arrest and inhibiting tumor invasion, adhesion and metastasis. Xin et al. isolated an acidic polysaccharide (CPPA) from the roots of CP and examined the effects of CPPA on tumor cell growth, invasion, and migration in vitro. They found that CPPA had a potent inhibitory effect on the invasion and migration

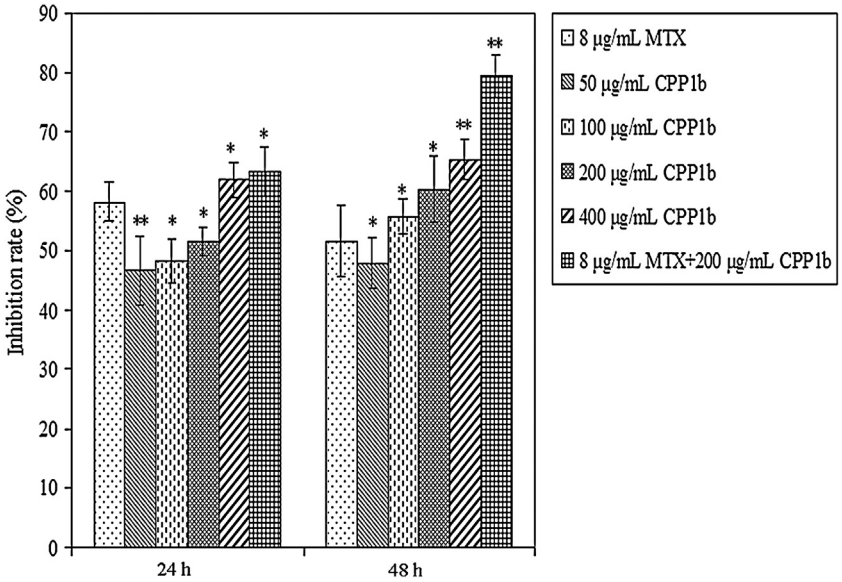


Fig. 6. The in vitro inhibition ratio of human lung adenocarcinoma A549 cells by CPP1b at different concentration. Values are means ± SD of five separated experiments. Significant differences compared to positive control group are designated as $^{*}p < 0.05$ and $^{**}p < 0.01$.

potential of human epithelial ovarian cancer HO-8910 cells in vitro (Xin et al., 2012). Therefore, it is reasonable to think that CPPA exerted direct cytotoxicity against HO-8910 cells. Fan et al. studied the relationship of the inhibition of cell migration to the structure of pectic polysaccharides from ginseng (WGPA) and found that the inhibitory effect of WGPA was related to the GalA content, and the fraction that contained the highest content of GalA (83.6%) exhibited significant inhibition (Fan et al., 2010). Therefore, we suggest that CPP1b may exert direct cytotoxicity against A549 cells, and the high content of GalA may play a vital role in this activity.

The immunoenhancement effect describes the ability of a compound to enhance host immune function, which has been considered the main or singular mechanism of some types of polysaccharides. The exact mechanism of the antitumor properties of CPP1b is worthy of further investigation because of its potential antitumor effect. Synergistic studies with other chemotherapeutic drugs have shown distinct improvements in the antitumor potential toward cancer treatments compared with single agents, which are mediated by enhancing the sensitivity of the tumor to the treatments and enhancing the immune response. In the present study, we examined the combined inhibition of CPP1b and MTX of A549 cells, and found that combined treatment was more effective than either drug in isolation, which suggested that CPP1b may exhibit synergistic activity with MTX against A549 cells. However, the exact synergistic effect of CPP1b and MTX on A549 cells awaits further investigation.

5. Conclusions

A pectic polysaccharide, CPP1b, was fractionated from extract of CP by DEAE-cellulose column chromatography. The molecular weight of CPP1b was approximately 1.45×10^5 Da and indicated a linear random coil conformation in 0.9% NaCl solution. Monosaccharide and uronic acid analysis revealed that CPP1b is composed of rhamnose, arabinose, galactose and galacturonic acid with a molar ratio of 0.25:0.12:0.13:2.51. The result of esterification showed that about 46.7 ± 0.4% of carboxylic groups in GalA residues existed as methyl ester. Methylation analysis showed that CPP1b contained 1,2-linked Rha*p*, terminal Ara, 1,2,6-linked Gal*p* and 1,4-linked Gal*p* at a molar ratio of 0.21:0.08:0.10:1.95. Based on the analysis of methylated 1D and 2D NMR, the backbone structure of CPP1b consists mainly of 1,4-linked α-D-Gal*p*A and 1,4-linked α-D-Gal*p*A6Me interspersed with rare 1,2-linked β-L-Rha*p*, 1,2,6-linked α-D-Gal*p* and terminal α-L-Ara*p*. TEM observations revealed that the ultrastructure of CPP1b is mainly composed of randomly distributed ovoid particles. SEM exhibited a surface with a sheet appearance. The results of antitumor activity assays showed that CPP1b possesses clear antitumor activities. Further studies are necessary to determine the detailed antitumor mechanism of CPP1b.

Conflict of interest statement

The authors have declared that no conflict of interest exists.

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