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Structural characterization and *in vitro* antitumor activity of a novel polysaccharide from *Taxus yunnanensis*

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ABSTRACT

The shrub, *Taxus yunnanensis* is famed as the source of the important anticancer drug, paclitaxel. But research on its polysaccharides contents has been scarce. The present research aimed to investigate the polysaccharide content of *T. yunnanensis* leaves and study the antitumor activities of isolated polysaccharide(s) using human tumor cells (K-562 and MCF). A novel heteropolysaccharide (TMP70W) was isolated and purified by anion-exchange and gel-permeation chromatography. Its molecular weight was 36.94 kDa and structural features were elucidated by partial acid hydrolysis, periodate oxidation–Smith degradation, methylation analysis, GC–MS, HPAEC-PAD, FT-IR, and NMR. The repeating unit of TMP70W had a backbone composed of (1→5)-linked-α-L-Ara*f*, (1→2,5)–linked-α-L-Ara*f*, and (1→6)–linked-β-D-Gal*p* with a branch of α-D-Glc*p*-(1→2)-α-D-Gal*p*-(1→ at the position of C-2 of arabinose. TMP70W displayed mild cytotoxicity against K562 cells with the IC_{50} value of 39.63 ± 2.37 μg/mL and inhibitory activity against MCF-7 cells (32.08 ± 0.39% at the concentration of 400 μg/mL) in a concentration-dependent manner.

1. Introduction

Taxus yunnanensis Cheng et L. K. Fu is an evergreen plant, which belongs to the Taxaceae family and endemic to south-western China and Burma. This shrub is the source of the important anticancer drug Taxol® (paclitaxel), which was first isolated from *Taxus brevifolia* (Wani, Taylor, Wall, Coggon, & McPhail, 1971). Paclitaxel possesses a unique mode of action against breast cancer, ovarian cancer, and other types of carcinoma (Kingston, Molinero, & Rimoldi, 1993). With the demonstrated clinical effectiveness of paclitaxel, extensive efforts have been taken to identify other members of the taxane group exhibiting potential antitumor activities. To date, many taxoids have been isolated from the leaves, stems, roots, and barks of *T. yunnanensi*, and some of them have exhibited significant clinical effects. However, the occurrence of adverse drug effects and multidrug resistances has impaired their further therapeutic application (Shinozaki et al., 2002). Consequently, later studies have begun to focus on more effective and less toxic compounds (Baldelli et al., 2004).

Polysaccharides have not been extensively explored in *Taxus* species as major bioactive components, although it is well known that polysaccharides play an important role in the growth and development of living organisms. In recent years, polysaccharides have been reported to exhibit a variety of biological activities such as antioxidant (Asker & Shawky, 2010), anti-inflammatory (Yu et al., 2004), antitumoral (Xin et al., 2012; Yin et al., 2010), antihyperlipidemic (Liu et al., 2012), antimutagenic (Liu et al., 2008), antiallergic (Tian, Che, Ha, Wei, & Zheng, 2012), antiglycation (Zhang, Wang, & Dong, 2011) and immunomodulatory (Fan, Ding, Ai, & Deng, 2012; Wang et al., 2011). In addition, some botanical polysaccharides have been commercially developed into important components of therapeutic drugs and skin care products (Deters, Dauer, Schnetz, Fartasch, & Hensel, 2001). In our previous work (Yin et al., 2010), TMP70S-1, one of the polysaccharides of *T. yunnanensis*, significantly inhibited the proliferation of HeLa and HT1080 cells in a concentration-dependent manner, indicating that the polysaccharides from *T. yunnanensis* could be developed as effective antitumor compounds apart from its taxoids.

As an extension of our ongoing screening for anti-cancer drugs, in the present study, TMP70W, a newly discovered water-soluble polysaccharide, was isolated from *T. yunnanensis* leaves. It was then purified, and its chemical characterization was determined and *in vitro* antitumor activity was assessed.

2. Experimental

2.1. Materials and chemicals

The leaves of *T. yunnanensis* were collected from the Lijiang Prefecture in Yunnan Province, China. The voucher specimens were

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deposited in College of Pharmacy, Jinan University, China and were identified by Dr. Rongmin Yu.

Vitamin C was bought from Puboxin Biotechnology Co., Ltd. (Beijing, China); hydrogen peroxide (H_2O_2) was purchased from Shanghai Chemical Reagent Co. (Shanghai, China); and glucose, phenol, and sulphuric acid were obtained from Guangzhou Reagent Co. (Guangzhou, China). All reagents used in this study were of analytical grade.

2.2. General methods

Total carbohydrate content of TMP70W was determined by the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Optical rotation was measured with a Jasco P-1020 polarimeter. Infrared (IR) spectrum was recorded on a JASCO Fourier transform (FT)/IR-480 spectrophotometer. Nuclear magnetic resonance (NMR) spectra were recorded with a Bruker Avance 400 instrument, and the sample was dissolved in deuterium oxide (D_2O). High-performance anion exchange chromatography (HPAEC) was analyzed on a Dionex ICS-2500 system, which was coupled with pulsed amperometric detector (PAD) and equipped with a Carbo PAC ^{TM}PA10 (2.0 mm × 250 mm) column. Gas chromatography–mass spectrometry (GC–MS) was conducted with a Hewlett Packard 5895 instrument using a fused-silica capillary column (length: 30 m, internal diameter: 25 mm) coated with a 0.25 μm film of DB-5. The ionization potential was 70 eV, and the temperature of the ion source was 220 °C. Protein was analyzed by the Bradford assay (1976).

2.3. Extraction, fractionation, and purification of polysaccharides

The dried powder of *T. yunnanensis* leaves (300 g) was treated twice with 1800 mL of petroleum ether for 12 h in a Soxhlet apparatus to remove lipid and pigment. The defatted meals were extracted with ethyl acetate:acetone (1:1, v/v) in a Soxhlet apparatus to get taxol (Wani et al., 1971). The residues were further extracted three times with 4000 mL of hot distilled water (85–90 °C) for 8 h each time. The combined aqueous extracts were filtered and concentrated under reduced pressure. The proteins in the extracts were removed by the Sevag reagent (1-butanol/chloroform, 1:4, v/v) (Navarini et al., 1999).

Two fractions of crude polysaccharides, named as P50 and P70, were obtained by graded ethanol precipitation at the final concentration of 50% and 70% of ethanol, respectively. P70 was decolorized with 40% H_2O_2 and dialyzed against distilled water for 48 h. The resulting polysaccharide solution was concentrated and lyophilized. Then, P70 (500 mg) was re-dissolved in distilled water, applied to a DEAE-Cellulose 52 chromatography column (length: 40 cm, internal diameter: 2.6 cm), and eluted with distilled water and a gradient of 0–0.5 M sodium chloride at a flow rate of 30 mL/h. This process was monitored by the phenol–sulfuric acid method (Dubois et al., 1956), and two fractions (P70W and P70S) were obtained. P70W was further purified by a Sephacryl S-100 HR column (length: 80 cm, internal diameter: 2.6 cm), and eluted with distilled water at a flow rate of 0.3 mL/min. A polysaccharide was obtained, which was dialyzed and lyophilized to give a white purified polysaccharide (named TMP70W).

2.4. Determination of molecular weight and homogeneity

The molecular weight of TMP70W was determined using a gel chromatographic technique (Yu et al., 2009). Sample solution (10 mg/mL) was applied to a Sephacryl S-300 HR column, which was equilibrated and eluted with distilled water at a fixed flow rate of 18 mL/h. This process was monitored by the phenol–sulfuric acid method, and the elution volume was recorded. The elution volumes of Blue Dextran (2000 kDa) and Glucose were termed as column void volume (Vo) and column volume (Vt), respectively. Different average molecular weights of standard dextrans, T_{500} (450 kDa), T_{70} (68.5 kDa), T_{40} (44.4 kDa) and T_{10} (10.4 kDa), was prepared according to the aforementioned methods and passed through the column to obtain their elution volume, V_e, respectively. K_{av} was calculated using the following formula: $K_{av} = (V_e - V_o)/(V_t - V_o)$. The calibration curve of Lg MW (molecular weight) of standard dextrans against their K_{av} was obtained (Lg MW = −4.2591 K_{av} + 6.7953, r = 0.9998). A solution of TMP70W (10 mg/mL) in distilled water was applied to the same column. The elution volume of TMP70W was then plotted in the same graph, and the molecular weight of TMP70W was determined.

TMP70W was dissolved in water to subsaturation. Two fractions, termed as T20 and T40, were obtained by graded ethanol precipitation at the final concentration of 20% and 40% of ethanol, respectively. T20 and T40 were centrifuged; freeze-dried and optical rotations were measured at the same condition. It is well known that every polysaccharide has different optical rotation, and every polysaccharide has different solubility in different low content aqueous ethanol (Qian, Chen, Zhang, & Zhang, 2009; Zhang, 1999). If T20 and T40 had the same optical rotation, the homogeneity of TMP70W was confirmed.

2.5. Monosaccharide analysis

TMP70W (5 mg) was hydrolyzed with 2 M trifluoroacetic acid (TFA) at 121 °C in a sealed-tube for 6 h (Yin et al., 2010). The excess acid was removed by co-distillation with methanol (MeOH) after the hydrolysis was completed. The hydrolysate was analyzed by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD).

2.6. HPAEC-PAD analysis

The hydrolysate (5 mg) was dissolved in pure water (1 mg/mL). Twenty microliters of this solution was used for ion-chromatography analysis by HAPEC-PAD of Dionex ICS-2500 System, eluted with a mixture of distilled water and 200 mM sodium hydroxide (NaOH) in the volume ratio of 92:8.

2.7. Infrared spectrum analysis

The infrared spectrum of TMP70W was determined using a JASCO FT/IR-480 spectrophotometer. The chromatographically purified TMP70W was grounded with potassium bromide powder and pressed into pellets for transformation IR spectra measurement at a frequency range of 4000–500 cm^{-1}.

2.8. Partial acid hydrolysis

TMP70W (30 mg) was hydrolyzed with 0.05 M TFA at 100 °C for 5 h. The hydrolysis product was evaporated by co-distillation with water to remove the excess acid. The residue was dialyzed in a small amount of distilled water for 48 h, and fraction A was obtained. The non-dialyzable part was hydrolyzed with 0.5 M TFA at 100 °C for 4 h to generate fraction B. The non-dialyzable part was hydrolyzed with 2 M TFA at 100 °C for 5 h to get fraction C. Then, the fraction A, B, and C were analyzed by HPAEC-PAD for the monosaccharide composition analysis.

2.9. Periodate oxidation and Smith degradation studies

TMP70W (15 mg) was allowed to swell overnight in distilled water (12.5 mL) and dispersed using a blender. Upon addition of 30 mM sodium periodate (12.5 mL), an immediate loss of viscosity

occurred. The solution was kept in the dark at room temperature, and 50 μL aliquots were withdrawn at 6 h intervals. The aliquots were diluted to 10 mL with distilled water and read in a spectrophotometer at 223 nm (Linker, Evans, & Impallomeni, 2001). Complete oxidation was identified with a stable absorbance. Consumption of periodic acid (HIO_4) was measured by a spectrophotometric method (Aspinall & Ferrier, 1957), and formic acid production was determined by titration with 0.0043 M NaOH. Ethylene glycol (2 mL) was added to terminate the experiment of periodate oxidation. The rest of the periodate product was exhaustively dialyzed against tap water (for 48 h) and distilled water (for 24 h). The solution was reduced with sodium borohydride ($NaBH_4$) (40 mg) overnight at room temperature and neutralized to pH 6.0–7.0 with 0.1 M acetic acid. After the solution was dialyzed and concentrated under vacuum, the product mixture was hydrolyzed with 2 M sulphuric acid at 100 °C for 8 h. Then, the hydrolysate was neutralized with barium carbonate and filtered. The obtained polysaccharide polyol was dissolved in pure water (1 mL) and analyzed by HAPEC-PAD under the same conditions as those used for the monosaccharide analysis.

2.10. Methylation analysis

Methylation analysis of TMP70W (5 mg) was performed by the method of Hakomori (1964). The methylated polysaccharide TMP70W was treated with 90% aqueous formic acid (3 mL) for 6 h at 100 °C in a sealed tube. After removal of the formic acid, the residues were hydrolyzed with 2 M TFA (2 mL) under the same conditions, and the hydrolysate was concentrated to dryness. The methylated sugars were reduced with $NaBH_4$, acetylated with acetic anhydride (Sassaki, Iacomini, & Gorin, 2005), and analyzed as the alditol acetates by GC–MS. The methylated sugar linkages were identified on the basis of relative retention time and fragmentation pattern (Yu et al., 2009). The molar ratios for each sugar were calibrated using the peak areas and response factors of the flame-ionization detector in GC.

2.11. NMR spectroscopy

^1H and ^{13}C NMR experiments were carried out at 400 MHz and 100 MHz on a Bruker Avance DPX-400 spectrometer, respectively. TMP70W (30 mg) was dissolved in 0.6 mL of D_2O. ^1H and ^{13}C NMR spectra were recorded at 60 °C. Acetone was used as an internal standard for the ^{13}C spectrum. The ^1H NMR spectrum was recorded by fixing the HOD signal at δ4.52. Chemical shift was expressed in ppm.

2.12. In vitro antitumor test

2.12.1. Cell lines and culture

Human breast adenocarcinoma cell line (MCF-7) and human chronic myeloblastic leukemia cell line (K562) (Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences) were cultured in Rosewell Park Memorial Institute 1640 medium (RPMI 1640), which was supplemented with heat-inactivated 10% fetal bovine serum, 0.2 mg/L L-glutamide, 1.0 mg/mL sodium bicarbonate, 100 units/mL penicillin, and 100 units/mL streptomycin in a humidified incubator at 37 °C and 5% carbon dioxide atmosphere. Cells were sub-cultured in the same medium under the same conditions after reaching 70–80% confluence and were maintained in the log phase for further experiments.

2.12.2. Cytotoxicity assay by MTT

Cytotoxicity was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric assay developed by Mosmann (1983). Cells were harvested from maintenance cultures in the exponential growth phase and were counted by a hemocytometer using Trypan Blue solution. The cell suspensions were dispensed in triplicate into 96-well culture plates at the density of 1×10^5 cells/mL. After incubation for 24 h, cells were treated with different concentrations of TMP70W. Cis-diaminodichloroplatinum was used as the positive control, and untreated cells were used as negative control. After an additional 72 h incubation period, the medium in each well was aspirated and replaced with 20 μl MTT working solution (5 mg/mL). The cells were incubated at 37 °C for 4 h, and the medium was then aspirated and replaced with 100 μL dimethyl sulfoxide to dissolve the formed formazan crystals. The culture plates were shaken for 10 min, and the absorbance of each well was read at 570 nm (Fatope, Zeng, Ohayaga, Shi, & Mclaughlin, 1996). All experiments were carried out in triplicate, and the data were presented in the form of mean ± SD. Inhibition ratio of tumor cell proliferation was calculated according to the formula below (Fan et al., 2012):

$$\text{Inhibition ratio}(\%) = \left(1 - \left(\frac{\text{OD}}{\text{OD}_0}\right)\right) \times 100\%$$

where OD and OD_0 are the absorbance of treated cells and untreated cells.

3. Result and discussion

3.1. Isolation, purification, and composition of polysaccharides

The yield of crude water-soluble polysaccharide from *T. yunnanensi* was 5.3%. Two crude polysaccharides, P50 and P70, were obtained by ethanol precipitation with yields of 3.4% and 1.9%, respectively. The water-soluble crude polysaccharide, named P70, gave two peaks on the DEAE-cellulose 52 column chromatography for purification. Peak I eluted by distilled water was further purified by Sephacryl S-100 HR column chromatography to get TMP70W. The structural characterization and anti-tumor activity of peak II, TMPS-1, has been reported in our previous work (Yin et al., 2010). The homogeneity of TMP70W was elucidated by the following tests: it was eluted as a single peak from gel-filtration chromatography on Sephacryl S-300 HR column and had the same optical rotation in different low content aqueous ethanol by Jasco P-1020 automatic optical polarimeter at room temperature.

TMP70W appeared as a white powder. Profile of TMP70W on Sephacryl S-300 HR appeared as a single elution peak. Average molecular weight of TMP70W was 36.94 kDa, and the total sugar content was 97.93%. HPAEC-PAD chromatogram profiles of standard monosaccharide mixture solution and hydrolysate of TMP70W were shown in Fig. 1. By comparison of hydrolysis products (Fig. 1(B)) with the standard monosaccharide (Fig. 1(A)), three monosaccharides, L-Ara, D-Gal, and D-Glc were identified in the hydrolysate of TMP70W, and their molar ratios were 2.27:2.28:1.05 by HPAEC-PAD.

3.2. Structural characterization of TMP70W

TMP70W has a negative response to the Bradford test, and no absorption at 280 nm and 260 nm in the ultraviolet spectrum indicated the absence of protein and nucleic acid.

The IR spectrum of TMP70W (Fig. 2) displayed the characteristic IR absorption bands of polysaccharides. The broadly stretched intense peak at 3405 cm^{-1} was due to the hydroxyl stretching vibration. A weak C—H group stretching band at 2938 cm^{-1} could also be observed. A band at 1051 cm^{-1} caused by non-symmetric C—O—C stretching vibration could likewise be observed. On the other hand, the absorption band at 873 cm^{-1} indicated that

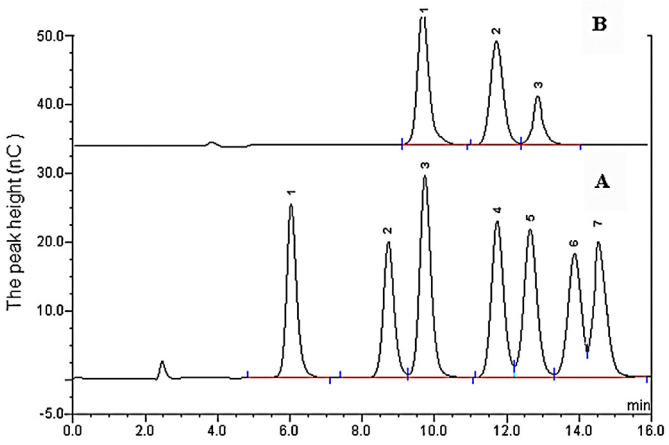

Fig. 1. (A) HPAEC-PAD chromatogram of standard monosaccharide mixture solution. 1. Fucose; 2. L-rhamnose; 3. L-arabinose; 4. D-galactose; 5. D-glucose; 6. D-mannose; 7. xylose; (B) HPAEC-PAD chromatogram of hydrolysate of TMP70W. 1. L-arabinose; 2. D-galactose; 3. D-glucose.

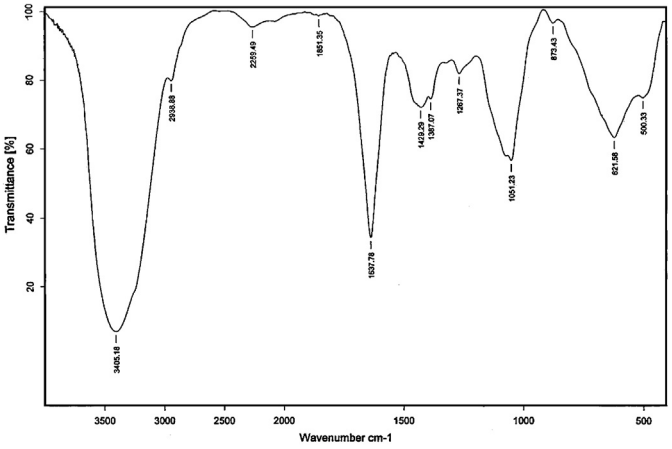

Fig. 2. IR spectrum of TMP70W.

TMP70W contained α-type glycosidic linkages in its structure (Yu et al., 2009).

To determine the backbone and branched chain in the structure of TMP70W, three fractions were obtained after partial acid hydrolysis of TMP70W: fraction A, fraction B and fraction C. Each hydrolysate was subjected to HPAEC-PAD analysis. The hydrolysis product of TMP70W hydrolyzed with 0.05 M TFA was dialyzed in a small amount of distilled water, and the material out of dialysis sack was co-distilled with MeOH and then dissolved in pure water to give fraction A, which had glucose:galactose molar ratio of 1.37:1. Therefore, the branched structure of TMP70W was composed of glucose and galactose, because these sugar residues could be obtained easily after hydrolysis. HPAEC-PAD analysis indicated that fraction B was composed of arabinose and galactose without glucose, and a molar ratio of arabinose and galactose in fraction C was 1.83:1. The above results indicated that arabinose and galactose might be the backbone of the TMP70W structure, and the branched chain might be composed of glucose and galactose.

The periodate-oxidated product of TMP70W was hydrolyzed and tested by HPAEC-PAD. This result showed the presence of arabinose and glycerol. The presence of arabinose indicated a part of arabinose is in (1→3)-linkage, (1→2,3)-linkage, (1→2,5)-linkage, (1→3,5)-linkage, or (1→2,3,5)-linkage that could not be oxidized by HIO_4. In addition, the presence of glycerol indicated that some residues were (1→)-linked, (1→2)-linked, (1→6)-linked, or (1→2,6)-linked. As galactose and glucose were not found in the hydrolyzed product, it could be inferred that galactose and glucose which could be oxidized were in (1→)-linkage, (1→2)-linkage, (1→6)-linkage, (1→2,6)-linkage, (1→4)-linkage or (1→4,6)-linkage. But, the absence of erythritol indicated that there was no (1→4)-linkage or (1→4,6)-linkage.

TMP70W showed abundant HIO_4 uptake during oxidation. The presence of formic acid in the product indicated the existence of 1-linked or 1,6-linked monosaccharides. The consumption of HIO_4 (0.3821 mmol) was twice that of the amount of formic acid (0.073 mmol), indicating the existence of large amounts of monosaccharides which were (1→4)-linked, (1→4,6)-linked, (1→2)-linked, or (1→2,6)-linked.

The fully methylated product of TMP70W was further analyzed by GC–MS. The polysaccharide showed the presence of five components (Table 1), namely 2,3-Me_2-Ara, 3-Me-Ara, 2,3,4,6-Me_4-Glc, 3,4,6-Me_3-Gal, and 2,3,4-Me_3-Gal. Based on the data available in Table 1, TMP70W was composed of (1→5)-linked-Ara, (1→2,5)-linked-Ara, (1→)-linked-Glc, (1→2)-linked-Gal, and (1→6)-linked-Gal, in molar ratio of 1.07:1.19:1.00:1.18:1.22 (about 1:1:1:1:1). Both the results of partial acid hydrolysis and methylation linkage analysis of TMP70W indicated that the residues of branch structure were composed of glucose and galactose, which linked to the oxygen of C-2 position of (1→5)-linked-Ara backbone. In addition, the molar ratios of arabinose, glucose, and galactose in fully methylated analysis were 2.26:1.00:2.40, which was in consistence with the result of HPAEC-PAD analysis as described above.

The ^1H and ^{13}C NMR spectra of purified TMP70W were shown in Fig. 3, respectively. Signals in NMR spectra were assigned based on component analysis, linkage analysis, and literature values (Yin et al., 2010; Yu et al., 2007, 2009). From the profile of ^{13}C spectra, the main →5)-α-L-Ara*f*-(1→ linkage was characterized by five strong signals at 109.8, 82.0, 77.4, 82.5, and 68.5 ppm; while the →2,5)-α-L-Ara*f*-(1→ linkage was characterized by signals at 107.9, 84.4, 76.1, 82.2, and 68.3 ppm. All of them originated from C-1, C-2, C-3, C-4, and C-5 of the L-arabinofuranosyl unit. In the proton NMR spectra, 33 signals of protons were recorded. The chemical shift at 5.27 ppm of anomeric H-1 indicated the presence of α form in the L-arabinofuranosyl unit. The signals identified at 100.1, 74.4, 78.5, 70.7, 78.8, and 62.9 ppm could be assigned to the C-1, C-2, C-3, C-4, C-5, and C-6 of the α-D-Glc*p*-(1→ unit. The chemical shift of H-1 at 5.50 ppm suggested the presence of α form in D-glucopyranosyl residue. The signals at 102.9, 72.4, 76.0, 69.6, 74.4, and 68.5 ppm in the ^{13}C spectra of TMP70W were related to the C-1, C-2, C-3, C-4, C-5, and C-6 of →6)-β-D-Gal*p*-(1→linkage; and the chemical shift at 99.4, 76.0, 70.1, 71.2, 72.1, and 61.6 ppm could be assigned to the C-1, C-2, C-3, C-4, C-5, and C-6 of →2)-α-D-Gal*p*-(1→ linkage. The H-1 signals at 4.52 and 5.38 ppm confirmed that the configurations of

Table 1
GC–MS analysis of the methylated products of TMP70W.

Retention time (min)	Methylated sugar	Mass fragments (*m/z*)	Linkage type	Molar ratios
13.63	2,3-Me_2-Ara	43, 45, 71, 87, 101, 117, 129, 161	5)-Ara-(1→	1.07
13.94	3-Me-Ara	43, 71, 87, 129, 189	→2,5)-Ara-(1→	1.19
14.42	2,3,4,6-Me_4-Glc	43, 71, 87, 101, 117, 131, 145, 161, 205	Glc-(1→	1.00
15.46	3,4,6-Me_3-Gal	43, 71, 99, 129, 189	→2)-Gal-(1→	1.18
17.14	2,3,4-Me_3-Gal	43, 71, 101, 129, 161, 189, 233	→6)-Gal-(1→	1.22

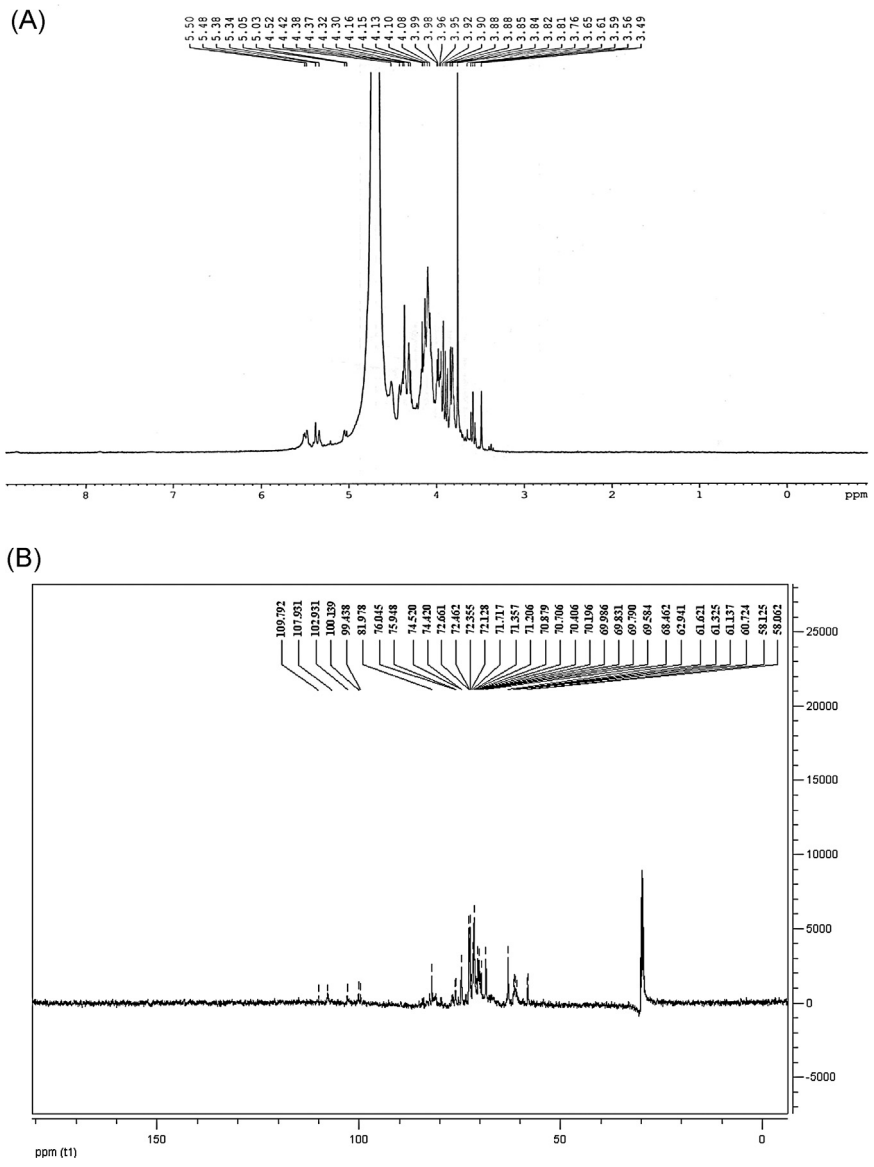

Fig. 3. ^{1}H (A) and ^{13}C (B) NMR spectrum of TMP70W.

D-galactopyranosyl residues were α and β forms, respectively (Errea & Matulewicz, 2003). The assignments of the proton and carbon signals were shown in Tables 2 and 3, respectively.

In ^{1}H NMR spectrum, the coupling phenomenon between the anomeric proton at δ5.50 belonged to α-D-Glc*p*-(1→ residue, and the anomeric proton at δ5.38 belonged to →2)-α-D-Gal*p*-(1→ unit. This observation indicated that α-D-Glc*p*-(1→ was linked with →2)-α-D-Gal*p*-(1→ residues. In combination with the results of partial acid hydrolysis, the residue α-D-Glc*p*-(1→2)-α-D-Gal*p*-(1→ was found to be the branched structure of TMP70W. In addition, the methylation analysis revealed that the backbone was composed of →5)-α-L-Ara*f*-(1→, →2,5)-α-L-Ara*f*-(1→, and →6)-β-D-Gal*p*-(1→ in molar ratio of 1.07:1.19:1.22 (about 1:1:1). Hence, the backbone structure of TMP70W was →5)-α-L-Ara*f*-(1→5)-α-L-Ara*f*-(2,1→6)-β-D- Gal*p*-(1→ residues. From the aforementioned results, it could be concluded that TMP70W was composed of repeating units having the possible structure shown in Fig. 4. To the best of our knowledge, the reported structure is novel, and this heteropolysaccharide has not been reported by others.

Table 2
The ^{1}H NMR chemical shifts of TMP70W.

Glycosyl residue	Chemical shift, δ (ppm)					
	H-1	H-2	H-3	H-4	H-5	H-6
→5)-α-L-Ara*f*-(1→	5.27	3.98	3.65	4.13	3.85	–
→2,5)-α-L-Ara*f*-(1→	N.d[a]	4.30	3.98	4.08	3.61	–
→6)-β-D-Gal*p*-(1→	4.52	3.65	3.76	3.81	3.90	3.98
α-D-Glc*p*-(1→	5.50	4.08	3.88	3.85	3.65	3.76
→2)-α-D-Gal*p*-(1→	5.38	3.95	4.08	4.10	4.37	3.81

[a] Not detected.

Table 3
The ^{13}C NMR chemical shifts of TMP70W.

Glycosyl residue	Chemical shift, δ (ppm)					
	C-1	C-2	C-3	C-4	C-5	C-6
→5)-α-L-Ara*f*-(1→	109.8	82.0	77.4	82.5	68.5	–
→2,5)-α-L-Ara*f*-(1→	107.9	84.4	76.1	82.2	68.3	–
→6)-β-D-Gal*p*-(1→	102.9	72.4	76.0	69.6	74.4	68.5
α-D-Glc*p*-(1→	100.1	74.4	78.5	70.7	78.8	62.9
→2)-α-D-Gal*p*-(1→	99.4	76.0	70.7	71.2	72.1	61.6

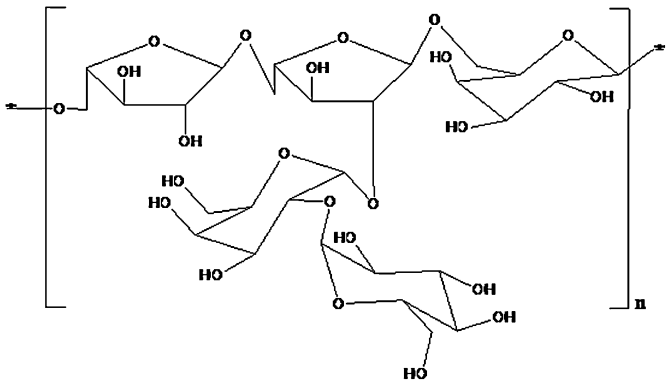

Fig. 4. Predicted structure of the repeating unit of TMP70W.

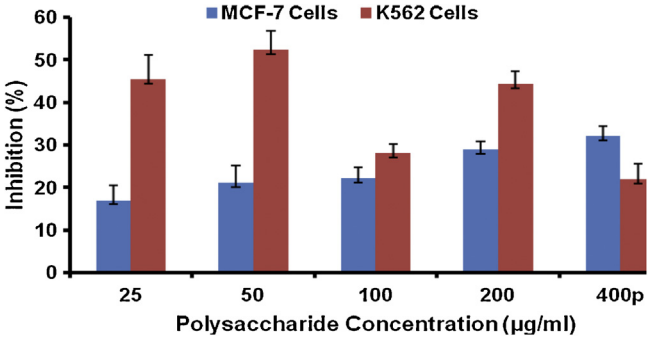

Fig. 5. Effects of TMP70W at different concentrations on MCF-7 and K562 cells by *in vitro* MTT assay. (The results were expressed as mean values ± SD).

Table 4
Effects of TMP70W at different concentrations on MCF-7 and K562 cells by *in vitro* MTT assay. (The results were expressed as mean values ± SD).

Concentration (μg/mL)	Inhibition (%)	
	MCF-7	K562
400	32.08 ± 2.39	21.92 ± 2.02
200	28.84 ± 1.91	44.34 ± 3.03
100	22.17 ± 2.04	28.01 ± 2.08
50	21.14 ± 1.99	52.37 ± 4.54
25	16.93 ± 1.42	45.33 ± 4.12

3.3. Cytotoxicity

To explore the biological activity of TMP70W, the inhibitory effect of TMP70W on human tumor cells (MCF-7 and K562) were investigated using MTT assay as shown in Fig. 5 and Table 4. The results showed that TMP70W influenced the proliferation of both the cell lines. TMP70W could inhibit the growth of K562 cells with an IC_{50} value of 39.63 ± 2.37 μg/mL. At concentrations of 100, 200, and 400 μg/mL, TMP70W suppressed the growth of MCF-7 cells with the inhibitory rates of 22.17 ± 2.46%, 28.84 ± 1.91%, and 32.08 ± 2.39%, respectively ($p < 0.05$). These results demonstrated that TMP70W had stronger cytotoxicity against K562 cells than against MCF-7 cells, and suggested that TMP70W could possess potential antitumor activity.

4. Conclusions

In the present study, a heteropolysaccharide (TMP70W) with a molecular weight of 36.94 kDa was isolated from *T. yunnanensis* leaves, and structurally characterized by chemical analysis, NMR, and IR spectroscopy. Per the experimental results, the repeating unit of TMP70W was found to be a backbone composed of →5)-α-L-Ara*f*-(1→5)-α-L-Ara*f*-(2,1→6)-β-D-Gal*p*-(1→ with a single branch of α-D-Glc*p*-(1→2)-α-D-Gal*p*-(1→ at the C-2 position of →2,5)-α-L-Ara*f*-(1→. The results of MTT assay demonstrated that TMP70W could mildly inhibit the proliferation of human tumor cells in a concentration-dependent manner. Further studies on the mechanism of the cytotoxicity of TMP70W are currently underway.

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