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Structural characterization and anti-tumor activity of a novel heteropolysaccharide isolated from *Taxus yunnanensis*

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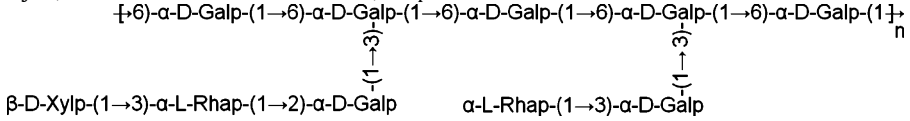
ABSTRACT

TMP70S-1, a novel water-soluble polysaccharide with molecular weight of 17.37 kDa, was isolated from *Taxus yunnanensis* Cheng et L. K. Fu by a combination of ion-exchange chromatography on the DEAE-Cellulose 52 and gel permeation on Sephacryl S-100. It was constituted of D-rhamnose, D-galactose, D-xylose in the molar ration of 2.01:6.74:1. According to total hydrolysis, periodate oxidation, methylation analysis, in combination with NMR studies, the predicted structure of TMP70S-1 was shown as follows:



The *in vitro* anti-tumor experiments of Hela and HT1080 cells by the method of MTT showed that TMP70S-1 could have a potent possibility for the selection as an anti-tumor agent.

1. Introduction

Plants from the genus *Taxus* are a rich source of biologically active diterpenoids belonging to the unique structure class of taxanes (Li, Huo, Zhang, & Shi, 2008), which is one of the most effective chemotherapeutic agents against a wide variety of tumors, especially ovarian and breast cancer (Yuan, Li, Hu, & Wu, 2001). Its unique mechanism of action has generated worldwide interest and extensive studies on taxol and its derivatives (Cordell, 1995).

Plant polysaccharides have drawn the attention of chemists and immunobiologists for their immunomodulatory and anti-tumor properties (Ghosh et al., 2008; Schepetkin & Quinn, 2006; Warrand et al., 2005). To date, no investigation has been carried out on polysaccharides that may account for anti-tumor activity of *Taxus yunnanensis*. Identification of polysaccharides is necessary in order to exploit their structural and functional properties. In this study, we report on the extraction, purification and structural identification of the major polysaccharide of *T. yunnanensis* using ion-exchange column and gel permeation column chromatography. In addition, the structural properties and *in vitro* anti-tumor activity of the major polysaccharide were also determined.

2. Materials and methods

2.1. Materials

T. yunnanensis Cheng et L. K. Fu was obtained from Guangdong Shanwei Biology Medicine Group Co., Ltd, Guangdong, China. The material (No. 080801) was identified by Professor R.M. Yu, College of Pharmacy, Jinan University, China.

The DEAE-Cellulose 52, DEAE-Sepharose, Sephacryl S-100 HR and Sephacryl S-300 HR were purchased from Phamacia. All other reagents were analytical grade.

2.2. General methods

The total sugar content of TMP70S-1 was determined by the phenol-sulfuric acid method using glucose as the standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). IR spectra were recorded with a Jasco FT/IR-480 spectrometer with KBr pellets. High performance anion exchange chromatography (HPAEC) was analyzed on a Dionex ICS-2500 system, coupled with pulsed amperometric detection (PAD), equipped with a Carbo PAC™ PA10 (2.0 mm × 250 mm) column. GC–MS was conducted with TRACE METM instrument, using a fused-silica capillary column (30 m × 25 mm) coated with DB-5. ^{13}C NMR spectra were recorded with a Bruker 400 instrument, and the sample was dissolved in D_2O.

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2.3. Extraction, Isolation and purification of TMP70S-1

Three hundred grams of the dry-up branch of *T. yunnanensis* were crushed and washed with acetone and ether to remove pigment and fat. The residues were extracted by distilled water at 90 °C for 4 h. The whole extract was centrifuged to remove the water-insoluble materials and the supernatant was collected, precipitated by 70% ethanol at 4 °C overnight. The precipitate was freeze-dried and deproteinated by Sevag method. The solution was dialyzed against distilled water for 24 h to remove low molecular weight materials (exclusion limit 3.5 kDa). The non-dialyzable portion was lyophilized and a brown crude polysaccharide was obtained (TMP70, yield: 5.6 g)

TMP70 was dissolved in distilled water, filtered through a 0.45 μm membrane filter and loaded on DEAE-Cellulose column (2.6 cm × 40 cm). The column was eluted with H_2O, followed by stepwise elution gradient of 0–0.5 M NaCl as the eluant at a flow rate of 0.5 ml/min. The crude polysaccharide TMP70S obtained by above procedure was further purified by gel permeation chromatography on a column of Sephacryl S-100 with water as the eluant. After several purification processes, the purified polysaccharide was obtained and named TMP70S-1.

2.4. Determination of molecular weight

The molecular weight of TMP70S-1 was determined by gel permeation chromatography on a column of Sephacryl S-300 HR (2.6 cm × 80 cm) with water as the eluant at a flow rate of 0.3 ml/min. The column was calibrated with standard T-series Dextran T-10, T-40, T-70, T-500 and blue Dextran, respectively. Elution volume of polysaccharide was plotted in the same graph, and the molecular weight of TMP70S-1 was determined.

2.5. Monosaccharide analysis

Five milligrams of TMP70S-1 were hydrolyzed with 2 M TFA at 121 °C for 6 h. The excess acid was removed by co-distillation with CH_3OH. The hydrolyzed product was divided into two parts. One part was examined by HPAEC-PAD. The other part was reduced with $NaBH_4$ at 20 °C overnight and the excess boric acid was decomposed by adding HOAc. The reduced product was acetylated with pyridine–Ac_2O (1:1, v/v) in a boiling water bath for 1 h converting to the alditol acetates (Dong, Ding, & Fang, 1998) and analyzed by GC–MS. The GC–MS analysis was carried out on a GC8060/TIC system (TRACE instrument, 70 eV) with DB-5 column (30 m × 0.25 mm × 0.25 μm) at a temperature program as follows: 150 °C (3 min) to 230 °C with a rate of 4 °C/min. The rate of N_2 carrier gas was 1.2 ml/min.

2.6. Partial acid hydrolysis

Thirty milligrams of TMP70S-1 were hydrolyzed with 0.05 M TFA at 100 °C for 5 h. The mixture hydrolysis product was evaporated by co-distillation with water to remove the excess acid and the residue was dialyzed in a small amount of distilled water for 48 h. The dialyzable part was concentrated, determined by HPAEC. The non-dialyzable part was hydrolyzed with 0.5 M TFA at 100 °C for 4 h. The non-dialyzable part was hydrolyzed with 2 M TFA at 100 °C for 5 h and the monosaccharide composition was determined by HPAEC.

2.7. Periodate oxidation

TMP70S-1 (15 mg) was oxidized with 0.030 M $NaIO_4$ (12.5 ml) in the dark for 120 h. The excess periodate was destroyed by adding 2 ml ethylene glycol and the consumption of $NaIO_4$ was measured by a UV spectrophotometer (Dixon & Lipkin, 1954). The yield of HCOOH was titrated with 0.00433 M NaOH. The solution was reduced with $NaBH_4$ at room temperature for 24 h and neutralized to pH 6.0–7.0 with HOAc. After dialyzed and concentrated, the mixture product was hydrolyzed with 2 M H_2SO_4 at 100 °C for 8 h. Then, the hydrolysis product was neutralized with $BaCO_3$ and filtered. The product of the oxidation of TMP70S-1 was analyzed by HPAEC for the monosaccharide composition analysis.

2.8. Methylation analysis

TMP70S-1 (3.0 mg) was methylated using the method described by Needs (Needs & Selvendran, 1993). The complete methylated polysaccharide was examined by IR spectroscopy with the lack of a hydroxyl peak. The methylated product was hydrolyzed, reduced and acetylated using the method described by Yu et al. (2009). The product of methylated alditol acetate was analyzed by GC–MS.

2.9. NMR spectroscopy

TMP70S-1 (30 mg) was dissolved in 0.6 ml of D_2O. The ^1H and ^{13}C spectra were recorded using a Bruker Avance 400 M Hz spectrometer at 80 °C. The ^{13}C NMR spectrum was used acetone as the internal standard.

2.10. Cell lines

Human cervix carcinoma Hela cells and Human fibroma carcinoma HT1080 cells were obtained from Institute of Biochemistry and Cell Biology, Shanghai, China. Hela and HT1080 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) in a humidified 5% CO_2 atmosphere at 37 °C.

2.11. Cell proliferation assay

The proliferation of Hela and HT1080 cells was determined by the colorimetric MTT assay. Hela and HT1080 cells were seeded at a density of 1×10^5 cells/well in 96-well plates for incubation 24 h. The concentrations of TMP70S-1 on every cell line were in the range from 12.5 to 400 μg/ml. The negative control was treated with the medium only and the positive control was treated with Cisplatin. Twenty microliter of MTT (5 mg/ml) was added in the medium 72 h later. After incubated at 37 °C for 4 h, the supernatant was aspirated and 100 μl of DMSO was added to each well. Absorbance was measured at 570 nm by a microplate reader.

3. Results and discussion

The crude polysaccharide was obtained from the *T. yunnanensis* by hot water extraction. The whole extract was precipitated in ethanol to 70% (v/v). The precipitate was then centrifugated, deproteinated and freeze-dried. The water-soluble crude polysaccharide, named TMP70, gave two peaks on the DEAE-Cellulose column chromatography for purification (Fig. 1). The major peak II eluted by NaCl was further purified by Sephacryl S-100 HR column chromatography (Fig. 2). According to Fig. 2, the purified fraction, named TMP70S-1, was obtained. The homogenicity of TMP70S-1 was elucidated by Sephacryl S-300 HR column chromatography as a single peak (Fig. 3), and its average molecular weight was 17.37 kDa. As showed in UV spectrum from 200 to 400 nm (Fig. 4), TMP70S-1 was free of protein and nucleic acid with the lack absorption peak in 280 and 260 nm.

Complete hydrolysis products of TMP70S-1 with 2 M TFA analyzed by HPAEC showed the presence of rhamnose, galactose and xylose. The GC–MS analysis of their alditol acetates showed the

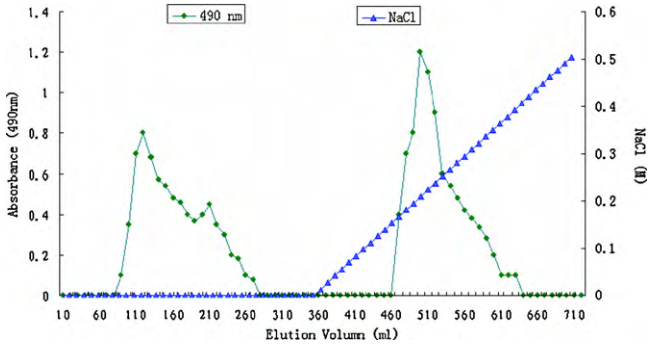

Fig. 1. Profile of TMP70 in DEAE-Cellulose column chromatography.

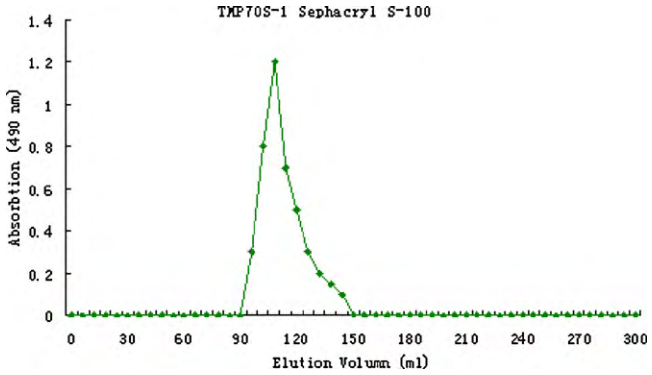

Fig. 2. Profile of TMP70S-1 in Sephacryl S-100 HR column chromatography.

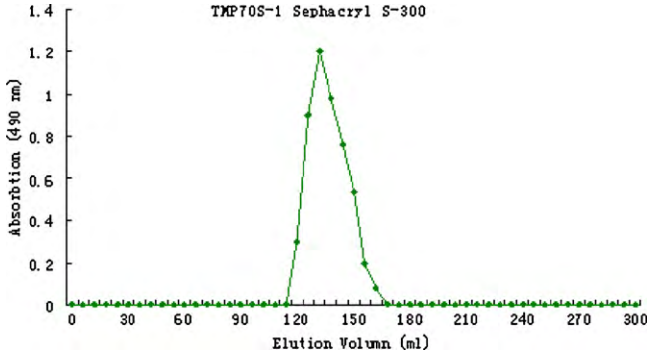

Fig. 3. Profile of TMP70S-1 in Sephacryl S-300 HR column chromatography.

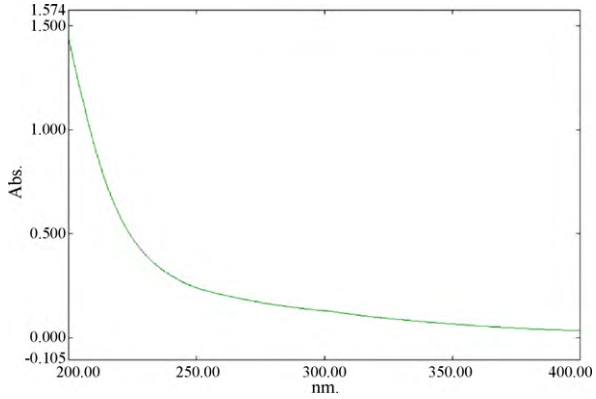

Fig. 4. The UV spectrum of TMP70S-1 from 200 to 400 nm.

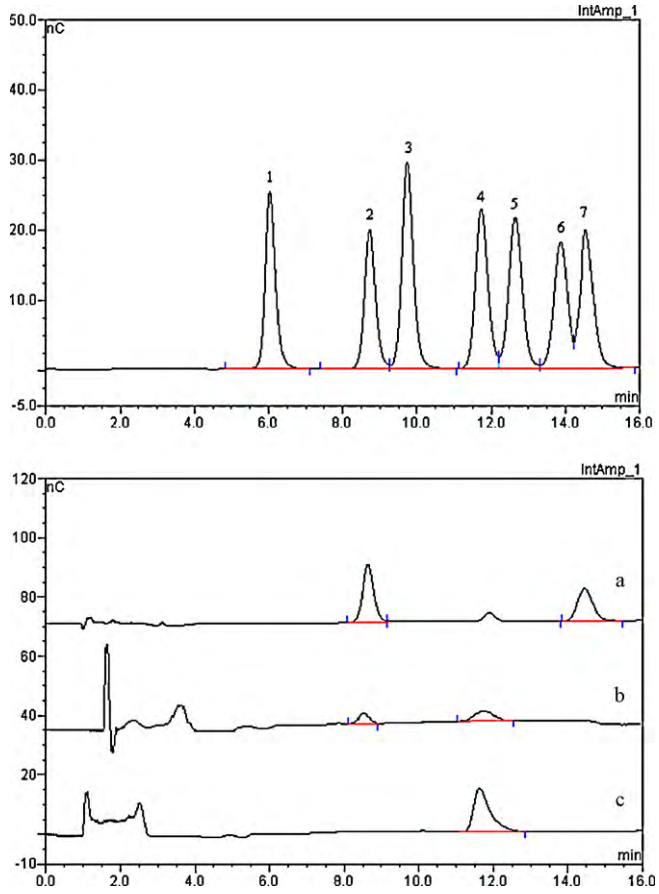

Fig. 5. (1) HPAEC-PAD chromatogram profile of standard monosaccharide mixture solution. 1. Fucose; 2. Rhamnose; 3. Arabinose; 4. Galactose; 5. Glucose; 6. Mannose; 7. Xylose. (2) HPAEC-PAD chromatogram profile of the partial acid hydrolysis products. (a) Partial acid hydrolysis with 0.05 M TFA out of the sack; (b) partial acid hydrolysis with 0.5 M TFA out of the sack and (c) partial acid hydrolysis with 2 M TFA in the sack.

rhamnose, galactose and xylose in the molar ratio of 2.01:6.74:1. Analysis of GC–MS also confirmed that galactose and xylose were in the D configuration. Xylose, rhamnose and galactose could be present at the branch chains of TMP70S-1 according to comparison of analysis results of partial acid hydrolysis products in 0.05 M TFA (Fig. 5(2a)) and 0.5 M TFA (Fig. 5(2b)) out of the sack with the result of the standard monosaccharide (Fig. 5(1)). The main backbone of TMP70S-1 was composed of galactose because only this monosaccharide was found out in the sack after 2 M TFA hydrolysis (Fig. 5(2c)).

TMP70S-1 was oxidized with 0.015 M $NaBH_4$ at room temperature in the dark for 120 h, the total consumption of $NaIO_4$ was 0.3393 mmol/mol of sugar residues and the production of formic acid was 0.012 mmol. The results showed that the non-reducing terminal residues or (1 → 6)-linked glycosyl bonds were in TMP70S-1 for the existence of formic acid. The products of the oxidation was reduced, hydrolyzed and analyzed by HPAEC. This result indicated the presence of glycerol, rhamnose and galactose. The presence of rhamnose and galactose indicated that some residues of rhamnose and galactose were (1 → 3)-linked, (1 → 2,3)-linked, (1 → 2,4)-linked, (1 → 3,4)-linked, (1 → 3,6)-linked or (1 → 2,3,4)-linked, which linkages cannot be oxidized. The absence of xylose in the oxidation products demonstrated that the residues of xylose were in all linkages that can be oxidized, namely (1→)-linkage, (1 → 2)-linkage, (1 → 6)-linkage and (1 → 2,6)-linkage. In addition, the presence of glycerol indicated that its residues were (1→)-linked, (1 → 2)-linked, (1 → 6)-linked, and (1 → 2,6)-linked.

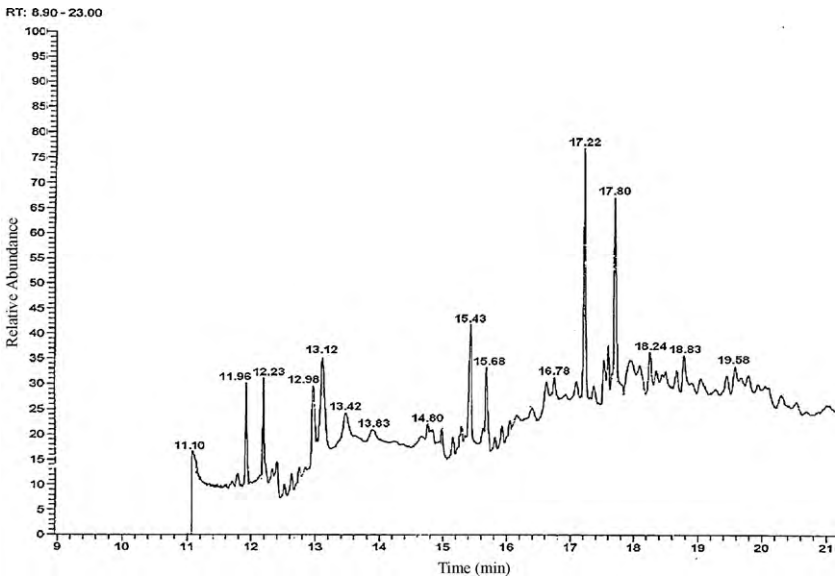

Fig. 6. GC profile of methylated TMP70S-1 on GC–MS.

The results of methylated polysaccharide showed that there are several major peaks from GC–MS analysis (Fig. 6), which might be cause by the methylated residues and volatile substances produced in the process of methylation. Seven types of residues derivatives, 2,3,4-Me_3-Rha, 2,4-Me_2-Rha, 2,3,4-Me_3-Xyl, 3,4,6-Me_3-Gal, 2,4,6-Me_3-Gal, 2,3,4-Me_3-Gal and 2,4-Me_2-Gal with the molar ratio of 1.02:1.07:1.15:1.12:1.00:2.97:2.03, were identified according to their mass fragments summarized in Table 1. The content of methylated rhamnose, xylose and galactose in the molar ratio of 2.09:1.15:7.12 was identical with the results of monosaccharide composition of complete acid hydrolysis. Both results of partial acid hydrolysis and methylation linkage analysis of TMP70S-1 indicated that (1 → 6)-linked galactose were the major residues of the backbone structure of TMP70S-1 and the terminal residues of xylose, rhamnose and galactose were joined to position C-3 of (1 → 6)-linked galactose. The result of molar ratio (2.97:2.03) of (1 → 6)-linked and (1 → 3,6)-linked galactose residues demonstrated that there were two branch residues in every five (1 → 6)-linked galactose residues.

The anomeric signals of each residue of TMP70S-1 in ^{13}C NMR spectrum (Fig. 7) and ^{1}H NMR spectrum (Fig. 8) were assigned by comparison with other NMR data from the literatures (Ganeshapillai, Vinogradov, Rousseau, Weese, & Monteiro, 2008; Nunes, Reis, Silva, Domingues, & Coimbra, 2008). There were seven signals from δ 98.0 to 105 ppm in the anomeric region. A signal at 101 ppm refers to the (1 → 6)-linked galactose residues, and the signal of anomeric proton of (1 → 6)-linked galactose in the ^{1}H NMR spectrum appeared at δ 5.02 ppm, which indicated the (1 → 6)-linked galactose residue was α-gylcosidic linkage. Similarly, the signals at δ 100.03 and 99.17 ppm were assigned to C-1 of (1 → 3)-linked and (1 → 2)-linked galactose residues, and the signals of anomeric protons appeared at δ 5.15 and 5.31 ppm, which indicated (1 → 3)-linked, (1 → 2)-linked galactose residues were both α-gylcosidic linkage. The detailed assignments of all the observed signals are shown in Table 2.

According to the literature data (Zhang, 1999), the C-1 signal of →3)-α-L-Rhap-(1→ was probable at δ 102.5 ppm when it linked at position O-2 of other residues, which indicated that the residues of →3)-α-L-Rhap-(1→ was an inter-residue correlation with →2)-α-D-Galp-(1→ residues. The C-1 signal of the terminal residue β-D-Xylp-(1→ was probable at δ 104.7 ppm when it linked at position O-3 of other residues, which indicated that the terminal residue β-D-Xylp-(1→ had an inter-residue correlation with →3)-α-L-Rhap-(1→ or →3)-α-D-Galp-(1→ residues. Moreover, if α-L-Rhap-(1→ had an inter-residue correlation with →3)-α-L-Rhap-(1→, the C-1 signal of α-L-Rhap-(1→ was probable at δ 102.8 ppm, which was not coincided with the results of C-1 signal of α-L-Rhap-(1→ (δ 103.09 ppm). Therefore, the β-D-Xylp-(1→ residue had an inter-residue correlation with →3)-α-D-Galp-(1→ residue. Based on the above analysis, the residues units of TMP70S-1 were composed of β-D-Xylp-(1 → 3)-α-L-Rhap-(1 → 2)-α-D-Galp-(1→ and α-L-Rhap-(1 → 3)-α-D-Galp-(1→.

By analyses of the results of methylation, periodate oxidation, acid hydrolysis, partial hydrolysis together with the results of NMR studies, the predicted structure of the novel polysaccharide TMP70S-1 is shown in Fig. 9.

Anti-tumor activity of TMP70S-1 was investigated against two kinds of human tumor cell lines, Hela and HT1080. Fig. 10 shows the effects of TMP70S-1 on the proliferation of Hela and HT1080 cells. At concentrations from 12.5 to 400 μg/ml. TMP70S-1 inhibited the proliferation of both tumor cell lines in a concentration-dependent manner.

Table 1
GC–MS results of methylation analysis of TMP70S-1.

Retention time	Methylated sugar	Mass fragments (*m*/*z*)	Type of linkage	Molar ratio
11.96	2,3,4-Me_3-Rha	43, 87, 101, 129, 143, 189, 203	Rha-(1→	1.02
12.23	2,4-Me_2-Rha	43, 59, 69, 75, 85, 99, 101, 117, 129	→3)-Rha-(1→	1.07
13.12	2,3,4-Me_3-Xyl	43, 101, 117, 161	Xyl-(1→	1.15
15.43	3,4,6-Me_3-Gal	43, 71, 99, 129, 189	→2)-Gal-(1→	1.12
15.68	2,4,6-Me_3-Gal	43, 45, 87, 101, 117, 129, 161	→3)-Gal-(1→	1.00
17.22	2,3,4-Me_3-Gal	43, 71, 101, 129, 161, 189, 233	→6)-Gal-(1→	2.97
17.80	2,4-Me_2-Gal	43, 87, 117, 129, 189	→3,6)-Gal-(1→	2.03

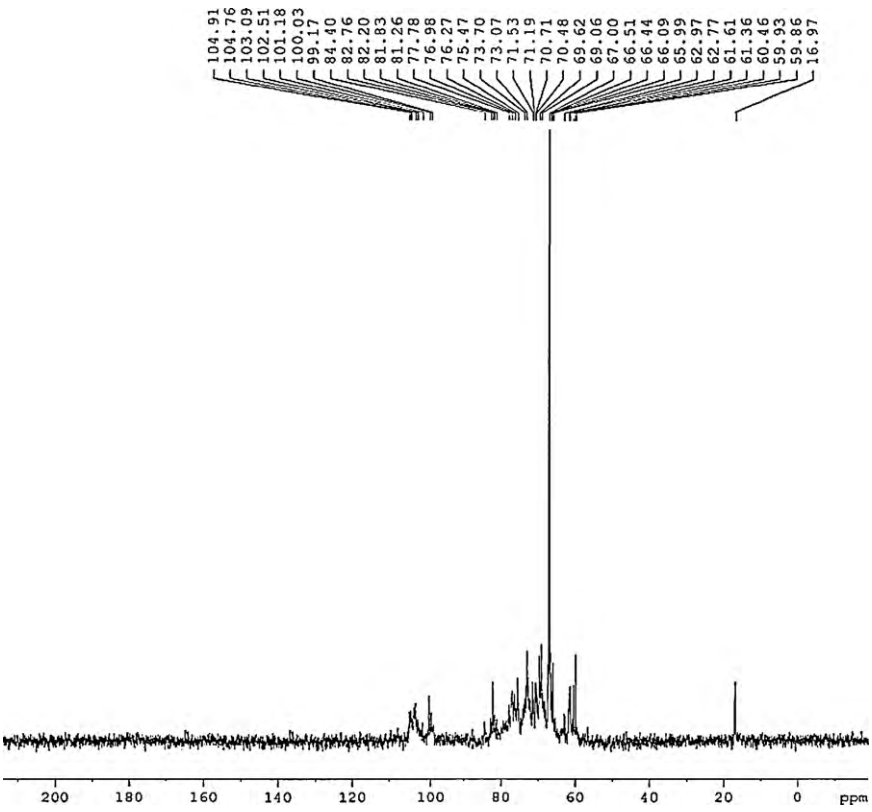

Fig. 7. ^{13}C NMR spectrum of TMP70S-1.

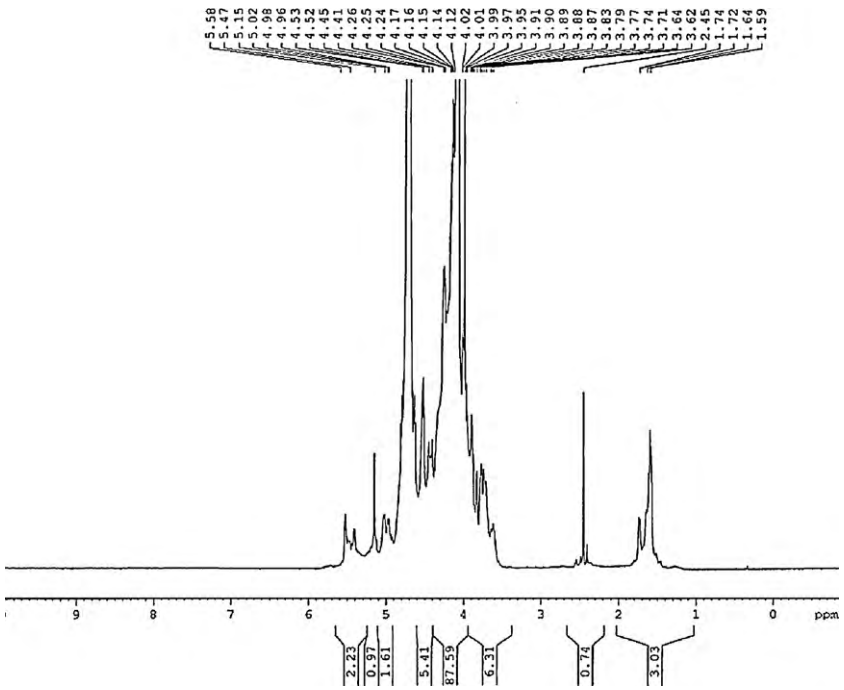

Fig. 8. ^{1}H NMR spectrum of TMP70S-1.

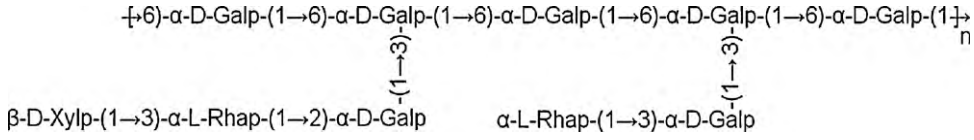

Fig. 9. Predicted structure of TMP70S-1.

Table 2
Assignment of ^{13}C NMR chemical shift values of TMP70S-1.

Sugar residues	Chemical shifts, δ (ppm)					
	C-1/H-1	C-2/H-2	C-3/H-3	C-4/H-4	C-5/H-5	C-6/H-6
-3,6)-β-D-Galp-(1→	104.91/4.50	71.19/3.64	82.76/4.17	69.62/3.64	76.27/3.89	61.61/3.95
β-D-Xylp-(1→	104.76/4.53	73.70/3.62	76.27/3.64	70.48/3.71	66.09/ND	–
α-L-Rhap-(1→	103.09/4.98	71.19/4.14	70.71/3.90	72.90/3.64	69.06/3.79	16.97/1.64
→3)-α-L-Rhap-(1→	102.51/5.47	70.71/4.15	79.52/3.99	72.40/3.61	69.62/3.91	16.97/1.74
→6)-α-D-Galp-(1→	101.18/5.02	72.90/3.95	71.53/4.14	72.40/4.01	72.20/4.26	69.06/3.83
→3)-α-D-Galp-(1→	100.03/5.15	71.4/4.01	79.4/4.02	70.3/4.26	75.2/4.17	65.4/3.77
→2)-α-D-Galp-(1→	99.17/5.31	75.47/3.99	70.71/4.02	71.19/4.12	72.40/4.26	62.77/3.79

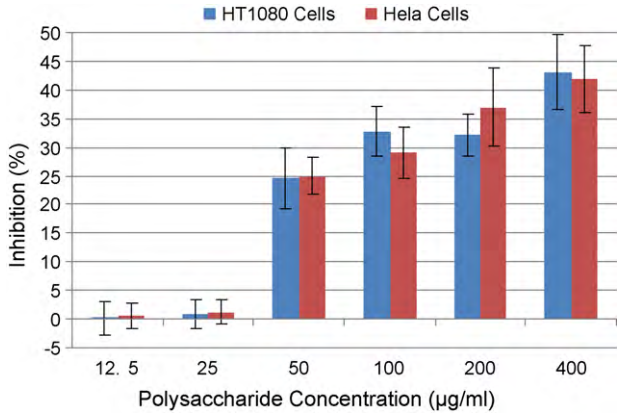

Fig. 10. Effect of TMP70S-1 at different concentrations on Hela and HT1080 cells by MTT assay.

In this study, the water-soluble polysaccharide TMP70S-1 was isolated from the *T. yunnanensis*. It was composed of rhamnose, xylose and galactose with the molecular weight of 17.37 kDa, and its repeating unit of structure was proposed as described above. According to the results of *in vitro* anti-tumor assay, TMP70S-1 was effective in the inhibition of proliferation of Hela and HT1080 cells.

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