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Purification, characterization and anti-proliferation activities of polysaccharides extracted from *Viscum coloratum* (*Kom.*) *Nakai*

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ABSTRACT

Three polysaccharides, VCP1, VCP2 and VCP3 were isolated from *Viscum coloratum* (*Kom.*) *Nakai* using DEAE-cellulose chromatography. VCP1 (32 KDa) was composed of glucose, galactose, arabinose, rhamnose and mannose, while VCP2 (280 KDa) and VCP3 (21 KDa) were consisted of glucose, galactose, arabinose, rhamnose, mannose, glucuronic acid and galacturonic acid. The optical rotation was measured at 20 + 1 °C. The characteristic absorptive bands of purified fraction were determined by FT-IR. ^{13}C NMR spectroscopy analysis showed that VCP1 was a neutral polysaccharide, and VCP2 and VCP3 were RG-I type pectin. The linkage patterns of VCP2 were evaluated by methylation analysis: 1,5-linked Ara*f*, 1,4-linked Gal*p*, 1,2-linked Rha*p*, and 1,2,4-linked Rha*p*. The degree of esterification was 50%. The anti-proliferation ability against HepG2 cells and HepG2.2.15 cells of VCP2 was stronger than VCP1 and VCP3. So the polysaccharides from *Viscum coloratum* (*Kom.*) *Nakai* could be used as potential natural sources with inhibiting tumor cells proliferation.

1. Introduction

In recent years, most studies focus on bioactive polysaccharides from organism due to the efficient anti-tumor activity and biosecurity. Polysaccharides are carbohydrates composed of more than 10 monosaccharides linked together through glycosidic bonds. Complex polysaccharides had been found bioactivities by Stimpel in 1984 (Stimpel, Proksch, Wagner, & Lohmann-Matthes, 1984). Then the chemical composition and bioactivity of polysaccharide have begun to be studied, they play key roles in the immune system (Zhang, Li et al., 2015), anti-tumor (Lin et al., 2013), anti-virus (Song et al., 2015), anti-ulcer (Austarheim et al., 2012) and anti-oxidant (Yang et al., 2016). More and more researchers are focusing on polysaccharide.

Viscum (also called "Hujisheng" in China) is a *Lorantbeceae* semi-parasite plant and more than 30 species widely distributed in the world, but 11 species distributed in China, *Viscum coloratum* (*Kom.*) *Nakai* is only included in Pharmacopoeia of the People's Republic of China. It is mainly found in Northeast China. There are two varieties, *Viscum coloratum* (*Kom.*) *Nakai f. Rubroauran tiacum Kitag* and *Viscum coloratum* (*Kom.*) *Nakai f. lutescens Kitag*, in addition, *Viscum album Lorantbeceae* is native to Europe, *Viscum album Lorantbeceae Coloratum* is originating in Koren which is a subspecies of *Viscum album Lorantbeceae*. In particular, mistletoe is commonly used in traditional herbal medicine to treat diseases as cancer, virus infection, hypertension, hyperlipidemia and arthritis (Yao et al., 2007). Its active components include small molecular compound of flavonoids compounds, organic acids and alkaloids and macromolecular compound such as phallotoxins, lectins, proteins and polysaccharides (Delebinski et al., 2015; Jiang et al., 2014; Rose et al., 2015). Polysaccharides extracted from *Viscum album* (L.) are reported to have the same constitution between branches and leaves, the main component is D-GalA, but the arabinogalactan is main component in fruits (Jordan & Wagner, 1986). However, the researches about *Viscum coloratum* (*Kom.*) *Nakai* mainly focus on alkaloids and lectins (Han, Hong, Kim, & Lyu, 2015), the reports of purification, characterization and anti-prolifeation activity of polysaccharides have still been less.

Therefore, in this study, three heteropolysaccharides designed as VCP1, VCP2 and VCP3 were isolated and purified from *Viscum coloratum* (*Kom.*) *Nakai.* Molecular weight (M*w*), monosaccharide composition, optical rotation (OR), ultraviolet (UV), fourier transformation infrared (FT-IR), nuclear magnetic resonance (NMR) and methylation analysis demonstrated the characteristics. Finally the potential effect on their anti-proliferation activities against HepG2 and HepG2.2.15 in vitro was also investigated.

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2. Materials and methods

2.1. Materials

The leaves of *Viscum coloratum* (*Kom.*) *Nakai* were collected from Tongjiang in Heilongjiang Province (China). All the samples were authenticated by Prof. Baojiang Zheng in College of Life Sciences, Northeast Forestry University, China. A voucher specimen was deposited at College of Life Sciences, Northeast Forestry University, China. Human hepatocellular carcinoma cell lines HepG2 cells and HepG2.2.15 cells with HepG2 cells transfected by a plasmid carrying two head-to-tail copies of HBV genome DNA were obtained from Harbin Veterinary Research Institute.

DEAE-cellulose, Sepharose CL-6B, a series of standard Dextrans of known M*w* (12, 50, 150, 470, 670 KDa) and standard monosaccharides of D-glucose (Glc), D-galactose (Gal), D-arabinose (Ara), L-rhamnose (Rha), D-mannose (Man), D-glucuronic acid (GlcA), D-galacturonic acid (GalA), bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, Mo, USA). Dulbecco's modified essential medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY, USA). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo (Shanghai, China). All other chemicals and reagents were of analytical grade.

2.2. Extraction isolation and purification of polysaccharides

The dry mistletoe leaves were ground into powder (40 mesh sieved), and the polysaccharides were extracted with hot-water and precipitated by ethanol. The mistletoe power (2 kg) was extracted with 95% ethanol (20 L) for 24 h to remove lipids. The dried residue was washed with 20 vol of distilled water at 95 °C for 2 h for three times and filtered. All filtrate was combined, concentrated by PEG dialysis (M*w* cut-off: 3500 Da) method and deproteinated by the Sevag method (Yao, Zhu, & Ren, 2016). The aqueous extract was centrifugated at 8000 rpm for 5 min to remove impurities. Then, the supernatant was precipitated adding ethanol to a final concentration of 80% and incubated for 24 h at 4 °C. Subsequently, the precipitate was obtained by centrifugation at 3000 rpm for 15 min and washed with anhydrous ethanol, acetone and diethyl ether for three times. Finally, crude *Viscum coloratum* (*Kom.*) *Nakai* polysaccharide (VCP) was obtained by lyophylization (176 g).

The crude VCP (20 g) was dissolved in distilled water (400 mL) and applied to a DEAE-cellulose column (8 × 20 cm) and eluted using 4 L of distilled water, 5 L of 0.2 mol/L, 0.35 mol/L NaCl, sequentially, at a flow rate of 13 mL/min to yield three main final fractions named VCP1, VCP2, and VCP3. The fractions were pooled, desalted, dialyzed, lyophilized and stored at −20 °C.Total carbohydrate content was measured by the phenol-sulfuric acid assay using Glc as the standard (Masuko et al., 2005). Protein content was assayed by Commassie Brilliant Blue G-250 and with Bovine serum albumin as the standard (Bradford, 1976). The uronic acid content assessed using *m*-hydroxydiphenyl-sulphuric acid assay with D-glucuronic acid as the standard (Filisetti-Cozzi & Carpita, 1991). Three polysaccharides were dissolved to 1 mg/mL solution and scanned from 200 to 600 nm with an UV spectrophotometer (Model SP-752, China).

2.3. Characterization of polysaccharides

2.3.1. Molecular weight determination

The M*w* of the polysaccharides was measured by gel filtration chromatography. The sample was dissolved in distilled water (5 mg/mL) and centrifugated at 10000 rpm for 5 min, the supernatant was applied to a Sepharose CL-6B column (1.5 × 100 cm). 0.15 mol/L NaCl was used as eluant and the flow rate was kept at 150 μL/min. A 3 mL aliquot was collected for each tube. The phenol-sulfuric acid method was used to determine the polysaccharide fractions. Dextran of different molecular weights (M*w*: 12, 50, 150, 470, 670 KDa) were used as standards for calibration.

The optical rotation was determined at 20 + 1 °C using Autopol IV-A21201 (Rudolph, Inc., USA).

2.3.2. Monosaccharide composition analysis

Monosaccharide composition was analyzed using high performance liquid chromatographic (HPLC) after precolumn-derivatization the hydrolysate with 1-phenyl-3-methyl-5-pyrazolone (PMP) as described previously (Chambers & Clamp, 1971; Strydom, 1994). Briefly, polysaccharide was hydrolyzed by trifluoroacetic acid (TFA, 2.0 mol/L) at 120 °C for 1 h in a sealed tube. Excess acid was removed at 45 °C for drying with adding a bit ethanol after hydrolysis. Subsequently, dry hydrolysate samples or monosaccharide standard aqueous solution were mixed with 0.5 mol/L methanol solution of PMP (500 μL) and 0.3 M aqueous NaOH (500 μL) for derivatization at 70 °C for 30 min, followed by centrifugation at 10000 rpm for 5 min. The mixture solution was added 50 μL of 0.3 mol/L HCl, the resulting solution was extracted with chloroform to remove the excess reagents. Finally, the aqueous layer was filtered through a 0.22 μm membrane for HPLC analysis, and 10 μL of the filtrate was injected into a DIKMAInertsil ODS-3 column (4.6 × 150 mm). The analysis of PMP-labeled monosaccharide was performed on a Shimadzu LC-10ATvp (SHIMADZU, Kyoto, Japan) HPLC system equipped with an UV detector (245 nm). The mobile phase was a mixture of PBS (0.1 mol/L, pH 7.0,) and acetonitrile (82:18). The elution was carried out at a flow rate of 1.0 mL/min.

2.3.3. Thermogravimetric analysis (TGA)

TGA was performed on Diamond 6300 (PerkinElmer, USA) according to described previously (Paramakrishnan, Jha, & Jayaram Kumar, 2015). 1660 mg of sample was placed in an aluminum oxide pan and heated within a temperature range of 30–600 °C at a heating rate of 10 °C/min under N_2 atmosphere to analyze the weight loss (TG) at the temperature.

2.3.4. FT-IR spectroscopy analysis

FT-IR spectroscopy was obtained using a Nicolet 6700 FT-IR spectrometer (ThermoScientific, USA) in the range of 4000–400 cm^{-1} with KBr pellets.

2.3.5. ^{13}C NMR analysis

20.0 mg of sample was dissolved in 500 μL of 99.8% D_2O. NMR spectra (^{13}C NMR) were recorded on a Bruker AV 600 MHz spectrometer (Germany).

2.3.6. Methylation analysis

VCP2 was methylated by the Bagchi and Jayaram Kumar method (Bagchi & Jayaram Kumar, 2016). Dry polysaccharide (20 mg) was dissolved with dimethyl sulfoxide (DMSO) and stirred still to obtain the clear solution. 240 mg of dry NaOH was added into the solution and stirred for 30 min. 3.6 mL of methyl iodide was added to the solution and stirred for 7 min. After adding 6 mL of distilled water to stop the reaction, the mixture was treated with the same volume chloroform and stirred for 30 min, and then washed with distilled water for three times. The methylated product was confirmed by FT-IR spectroscopy. Subsequently, the methylated sample was hydrolyzed by treatment of 85% formic acid solution (1 mL) at 100 °C for 4 h and TFA (2 mol/L, 2 mL) at 100 °C for 6 h, then it was co-distilled with methyl alcohol, anhydrous ethyl alcohol and distilled water to remove the excess acid, respectively. The hydrolysate was reduced with $NaBH_4$ overnight and then acetylated with 500 μL of acetic anhydride and pyridine at 100 °C for 2 h,