CHARACTERIZATION OF A NEW GLUCOMANNAN FROM EREMURUS SPECTABILIS ROOTS

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Abstract- A novel glucomannan with molecular weight of 47.5 kDa was obtained from the roots of Eremurus spectabilis (serish) by warm-water extraction, ethanol precipitation, deproteinization and lyophilization. The crude polysaccharide was purified through DEAE-cellulose A52 and Sephadex G-100 columns to obtain a homogeneous polysaccharide. It was composed of D- mannose and D- glucose in 2.9:1 molar ratio. Chemical and spectroscopic analyses indicate that polysaccharide possessed a backbone of (1→4)-linked ß-D-glucopyranosyl and ß-D-mannopyranosyl, branched with ß-(1→4,6)-D-mannopyranose and terminate with ß-D-mannopyranose.

Index Terms- Glucomannan, Eremurus spectabilis, Structure, Plant polysaccharide.

I. INTRODUCTION

Roots of medical plants are important resources of interesting bioactive polysaccharide, many of which have been reported to possess various biological functions. For instance, aglucan isolated from the roots of Rubus crataegifolius Bge. exhibits strong immuno-logical activity [1] and an arabinoglucogalactan isolated from Panax notoginseng root displays antioxidant activity [2].

The genus Eremurus, popularly called "serish" in Iran, belongs to the Liliaceae family. Eremurus spectabilis is one of the most important species from this genus. It grows very well in South and Central Asia, including Iran, West Pakistan, Afghanistan, Iraq, Turkey, Palestine, Lebanon, Syria and Caucasus [3]. The people locally use the hypogeal organs (roots) of this plant to cure jaundice, liver disorders, stomach irritation, pimples and bone fractures and even as a glue for industrial application [4]. There is relatively little information referring to the isolation, purification and structural determination of the water-soluble polysaccharides from Eremurus spp. [5-13], and there is none at all on the purification and structural elucidation of polysaccharides from the roots of E. spectabilis. Since structure and functions are closely related, an in-depth study of the structure of these polysaccharides would be of interest. Therefore, in this work, we specifically focused on the isolation and structural characterization of a water-soluble polysaccharide ESPS-1 from E. spectabilis.

II. METHODOLOGY

A. Chemicals and plant materials

The roots of E. spectabilis were collected by the authors in April 2014 in Shahr-e Kord, Chahar Mahall va Bakhtiari province, Iran and transported to the laboratory, dried and stored at room temperature. Taxonomic identification was done by Prof. Valiollah Mozaffarian, botanical taxonomist. The roots were scraped with a knife to remove the natural powder that coats them. The scraped samples were further cleaned by washing them in water and drying them. The samples were then milled into powder using a laboratory grinding machine. DEAE-Cellulose A52 and Sephadex G-100 were purchased from the Pharmacia Co. (Uppsala, Sweden). Dextrans of different molecular weights and pure monosaccharide standards were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Trifluoroacetic acid (TFA) was purchased from Fluka. Aqueous solutions were prepared with ultra-pure water from a Milli-Q water purification system (Millipore, Bedford, MA, USA). All other reagents used in this study were of analytical grade.

B. General analysis

Concentrations were performed under diminished pressure at around 45 °C (Heidolph Laborota 4000 efficient rotary evaporator, Germany). The products were dried by vacuum freeze drying (Christ Alpha 2-4 freeze drier, Germany). Total carbohydrate content of the polysaccharide was determined by the phenol-sulfuric acid colorimetric method, using D-glucose as the standard at 490 nm [14]. In addition, protein in the polysaccharide was quantified according to the Bradford method [15], using bovine serum albumin (BSA) as the standard. Specific optical rotations were measured at 20 °C using a Perkin–Elmer 343 polarimeter at 589 nm. Ultraviolet-visible absorption spectra were recorded with a Varian Cary100-Bio UV/visible spectrophotometer (USA). Infrared spectra were recorded using a Fourier transform infrared spectrophotometer (FT-IR, Nicolet 5700 Instrument, Thermo Company, Madison, USA) with KBr pellets in the frequency range 4000 to 400 cm^{-1}. Gas chromatography (GC) was performed on a Varian 3400 instrument (Hewlett-Packard Component, USA) equipped with a DM-2330 capillary column (30 m × 0.32 mm × 0.20 µm) and flame-ionization detector (FID). The column temperature was kept at 120 °C for 2 min, then increased to 250 °C (maintained for 3 min) at a rate of 8 °C/min. N_2 was used as the carrier gas at a

flow rate of 1.2 ml/min. The injector and detector heater temperature were 250 and 300 °C, respectively. Gas chromatography-mass spectrometry (GC-MS) was done on a HP5890 (II) instrument (Hewlett-Packard Component, USA) with an HPS quartz capillary column (25 m × 0.22 mm × 0.20 μm), and at temperatures programmed from 120 °C (maintained for 2 min) to 260 °C (kept for 40 min) at a rate of 15 °C/min.

C. Extraction and purification of the polysaccharide

Polysaccharide was extracted and purified by the method of Jahanbin et al. [16] as described early. The powdered roots of E. spectabilis were soaked with 3 volume of 95% ethanol for 10 h under reflux to remove the pigments and small lipophilic molecules, and the supernatant was removed. The residue was then extracted three times with warm water (50 °C), each time for 10 h. All water-extracts were combined, filtrated, concentrated to 1/3 volume under reduced pressure, and precipitated with 95% ethanol (1:4, v/v) at 4 °C for overnight. The precipitate was collected by centrifugation (5000g) and deproteinated by Sevag reagent (1-butanol/chloroform, v/v = 1:4) [17]. Finally the supernatant was lyophilized to give crude E. spectabilis polysaccharides (CESP). CESP was redissolved in deionized water and forced through a filter (0.45 μm), then loaded onto a column (2.6 cm × 30 cm) of DEAE-Cellulose A52. After loading with sample, the column was eluted with gradient NaCl aqueous solution (0–1 M), and the procedure was monitored by the phenol-sulfuric acid method mentioned above. A major polysaccharide fraction (ESPS) was further purified on a Sephadex G-100 gel filtration column (1.6 cm × 70 cm), and eluted with deionized water, at a flow rate of 8 ml/h. The main polysaccharide fraction was collected and lyophilized to obtain white purified polysaccharide named, as ESPS-1 and used for further study.

D. Monosaccharide composition analysis

The monosaccharide composition of ESPS-1 was analyzed by GC. ESPS-1 was hydrolyzed with 2 M TFA at 120 °C in a sealed tube for 2 h. The excess acid was completely removed by distilled water. Then the hydrolyzed products were reduced with $NaBH_4$, followed by neutralization with dilute acetic acid and evaporated at 45 °C. They were then co-distilled with methanol to remove the excess boric acid. The reduced products (alditols) were acetylated with 1:1 pyridine–acetic anhydride in a water bath for 1 h at 90 °C to give the alditol acetates. Alditol acetates of authentic standards with myo-inositol as the internal standard were prepared and subjected to GC analysis separately in the same way [18].

E. Partial acid hydrolysis

ESPS-1 was hydrolyzed with 0.05 M TFA at 80 °C for 16 h. After cooling, TFA was evaporated with methanol under reduced pressure. The hydrolyzed ESPS-1 was centrifuged to remove the precipitate, and the supernatant was dialyzed against distilled water for 24 h in a dialysis bag (molecular weight 3.5 kDa cut off). After dialysis, the fraction removed was collected. Ethanol was added to the solution in dialysis sack, and the precipitation and supernatant were collected [19]. All fractions were analyzed by GC as described above.

F. Periodate oxidation - Smith degradation

ESPS-1 was oxidized with 0.04 M NaIO4 at 4 °C in the dark and its absorption was monitored at 223 nm every 4 h. After the oxidation reaction was complete (96 h), the excess $NaIO_4$ was destroyed by adding ethylene glycol. The amount of $NaIO_4$ consumption was calculated according to the decrease in absorbance at 223 nm [20], and formic-acid production was determined by titration with 0.053 M NaOH. The periodate reaction mixture was extensively dialyzed against tap water and distilled water for 48 h and reduced with $NaBH_4$ (50 mg) for 12 h at 25 °C. The residue was subjected to complete hydrolysis with 2 M TFA. The acid was removed by co-distillation with methanol under vacuum. Finally, the products were acetylated and analysed by GC [18].

G. Methylation analysis

Methylation analysis was performed according to the method of Needs and Selvendran [21]. The methylated products were extracted by chloroform and examined by IR spectroscopy. The absence of the absorption peak corresponding to hydroxyl group (3200–3700 cm^{-1}) indicated complete methylation. The methylated products were hydrolyzed with 2 M TFA at 120 °C for 2 h in a sealed tube, and excess acid was evaporated by co-distillation with distilled water. The hydrolyzed product was reduced with $NaBH_4$ (2 h, room temperature) and finally acetylated with 1:1 acetic anhydride-pyridine. The partially methylated alditol acetates were analyzed by GC–MS.

H. Nuclear magnetic resonance (NMR) spectroscopy

^{13}C and ^{1}H NMR spectra of the polysaccharide were recorded at 27 °C on a Bruker Avance 500 MHz NMR spectrometer (Brucker, Rheinstetten, Germany) operating at 126 MHz for ^{13}C NMR and 500 MHz for ^{1}H NMR. DSS was used as the internal standard and delay time was 2 s. Chemical shifts are given in ppm. The freeze-dried polysaccharide was exchanged with deuterium by lyophilisation with D_2O and then examined in 99.9% D_2O [22].

III. RESULTS & DISCUSSIONS

In this study, the water-soluble crude polysaccharide, named CESP, isolated (as a light-yellow powder) from the roots of E. spectabilis by warm-water extraction followed by ethanol precipitation, deproteinization and lyophilization. The extraction yield of CESP was about 11% of the dried material. CESP was purified with DEAE-cellulose A52 and Sephadex G-100 gel-filtration columns. The main fraction (ESPS-1) was collected and lyophilized for further analysis of structure.

ESPS-1, which appeared as a white powder, showed a negative response to the Bradford method and had no

absorption at 280 and 260 nm in the UV spectrum, indicating the absence of protein and nucleic acid. The optical rotation of ESPS-1 was $[\alpha]_D^{20}$ -36.3° (c 1.0, H_2O). This value of optical rotation (-36.3°) suggested the dominating presence of ß-form glycosidic linkages in ESPS-1. The GPC profile (Fig. 1) showed a single and symmetrically narrow peak, indicating that ESPS-1 was a homogeneous polysaccharide. The average molecular weight of ESPS-1 was about 47.5 kDa according to the calibration curve with standard dextrans. Total carbohydrate content of ESPS-1 was 99.2%, as determined by the phenol-sulfuric acid method. GC analysis (Fig. 2) showed ESPS-1 was composed of two kinds of monosaccharides, namely Mannose (Man) and glucose (Glc) with a relative molar ratio of 2.9:1.0. The results above indicate that the water soluble polysaccharide extracted from the roots of E. spectabilis is a glucomannan-type polysaccharide.

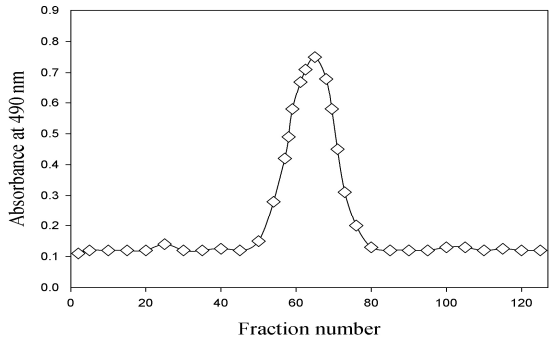

Fig. 1. The gel permeation chromatography of polysaccharide fraction (ESPS-1) on Sephadex G-100.

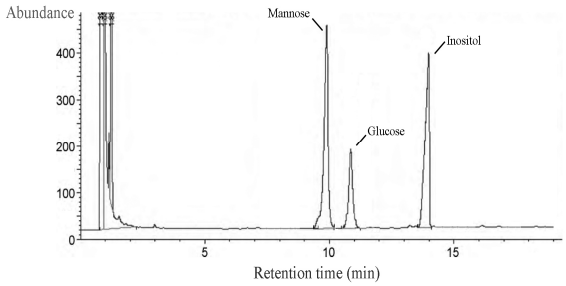

Fig. 2. Gas chromatogram of ESPS-1 hydrolyzed by TFA.

FT-IR spectroscopy is used to study the carbohydrates due to its ability to identify main functional groups of plant polysaccharides. As shown in Fig. 3, the IR spectrum of ESPS-1 revealed a typical major broad stretching peak around 3421 cm^{-1} for the hydroxyl group, and the small band at around 2912 cm^{-1} was attributed to the C–H stretching of CH_2 groups. The broad band at 1631 cm^{-1} was due to the associated water. Polysaccharides have specific bands in the 1200-950 cm^{-1} region (i.e., the so-called fingerprint region), where the position and intensity of the bands are specific for each polysaccharide, allowing its possible identification. The bands at 1457 and 1375 cm^{-1} are assigned to C–H vibrations of polysaccharides. The C–O ether bond showed stretching at about 1140 cm^{-1} while the C–O alcohol bond shown stretching at 1077 cm^{-1}. The peak at around 893 cm^{-1} is characteristic for ß-D-mannose and the band at 871 cm^{-1} indicated β-configurations of the sugar units existing in the polysaccharide [23]. On the basis of the aforementioned results, it can be concluded that ESPS-1 is composed of β-configurations in pyranose-form sugars. There was no absorption at 1250 and 1735 cm^{-1}, indicating no existence of acetyl groups in the polysaccharide structure [24].

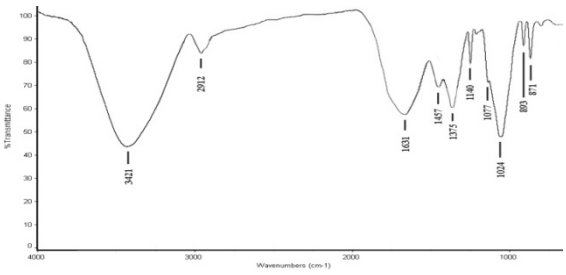

Fig. 3. FT-IR spectrum ESPS-1.

All the products after partial acid hydrolysis of ESPS-1, including ESPS-1a (precipitation after hydrolysis), ESPS-1b (precipitation in the dialysis sack), ESPS-1c (supernatant in the dialysis sack), and ESPS-1d (fraction out of dialysis sack), were subjected to GC analysis. From GC results we can conclude that Man and Glc in ESPS-1a and ESPS-1b comprised the backbone component of ESPS-1, and Man in ESPS-1c and ESPS-1d existed as the branched or terminal residues of ESPS-1.

On periodate oxidation, $NaIO_4$ consumption and formic acid production of the polysaccharide were 1.14 mol/mol sugar residue and 0.13 mol/mol sugar residue respectively, indicating the existence of small amount of monosaccharides which are 1→ linked or (1→6)-linked. The fact that the amount of periodate consumption was more than eight times the amount of formic acid (>0.13 mol × 8) demonstrated the existence of large amounts of (1→4) or (1→2)-linked sugar residue. The periodate-oxidized products of ESPS-1 were fully hydrolyzed and examined by GC. No Man and Glc were observed, and large amounts of erythritol and some glycerol were obtained, demonstrating that Man and Glc were all linkages that can be oxidized by periodate, namely 1→linkage, (1→6)-linkage, (1→2)-linkage, (1→4)-linkage, (1→2,6)-linkage and (1→4,6)-linkage.

No precipitates in the sack and no substances in the supernatant of the sack were observed on Smith degradation results. This finding suggests that the linkages of the backbone of ESPS-1 should be oxidized completely by HIO_4.

Methylation analysis by GC–MS was employed to determine the types and ratios of glycosidic linkages of monosaccharide residues in ESPS-1. As summarized in Table I, ESPS-1 showed the presence of four components, namely 2,3,4,6-Me_4-Man, 2,3,6-Me_3-Glc, 2,3,6-Me_3-Man and 2,3-Me_2-Man in

molar ratios of 0.96:2.13:4.21:1.02 (about 1:2:4:1). These molar ratios were in good agreement with the overall monosaccharide composition of ESPS-1 obtained from GC analysis. The above-mentioned results were in accordance with the results from partial-acid hydrolysis, periodate oxidation and Smith degradation. The methoxyl groups were not observed at C-5 which suggested that all monosaccharides were present in the pyranose form. This result confirmed the prediction made by FT-IR analysis regarding the presence of pyranose-form sugars in ESPS-1.

Table I. GC–MS results of methylation analysis of ESPS-1.

Methylated sugar	Molar ratio	Mass fragments (m/z)	Type of linkage
2,3,6-Me_3-Glc	2.13	43,45,71,87,99,101,113,117,129,161,173,233	→4)-Glc-(1→
2,3-Me_2-Man	1.02	43,85,87,101,117,127,142,159,161,201,261	→4,6)-Man-(1→
2,3,6-Me_3-Man	4.21	43,45,71,87,99,101,113,117,129,161,173,233	→4)-Man-(1→
2,3,4,6-Me_4-Man	0.96	43,45,71,87,101,117,129,145,161,205	Man-(1→

With results from methylation analysis, which were consistent with the results from partial-acid hydrolysis, periodate oxidation and Smith degradation, it could be concluded that 2,3,6-Me_3-Man (1,4-linked Man) and 2,3,6-Me_3-Glc (1,4-linked Glc) were major components of the backbone structure; part of Man was distributed in branches; and residues of branches terminated with Man.

NMR spectroscopy was used to complete the structural characterization of ESPS-1. In the proton spectrum, the signals at 4.73 and 4.51 ppm were easily assigned to anomeric protons of β-D-mannopyranose (β-D-Manp) and β-D-glucopyranose (β-D-Glcp) residues, respectively. The absence of signals at d 2.1–2.2 ppm indicated that there is no acetyl group in the polysaccharide structure. These results were in good agreement with the results from FT-IR.

Fig. 4 shows the ^{13}C NMR spectrum of ESPS-1. Based on the data available in the literature [25], the resonances in the region of 101.6 and 104.1 ppm were attributed to the anomeric carbon atoms of β-D-Manp, and β-D-Glcp respectively. The presence of the C-1 signal indicated that all sugar should be in the pyranose form, as the resonance of furanose form should be around 107 to 109 ppm. The signals at 78 ppm and 80.2 ppm were attributed to the C-4 of 1,4- and 1,4,6-linked Manp residues, and to the C-4 of 1,4-linked Glcp residues, respectively. No signals were observed at 20-22 ppm and 171-175 ppm for acetyl groups.

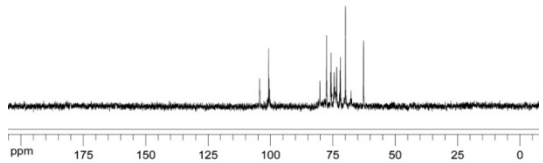

Fig. 4. ^{13}C NMR spectrum of ESPS-1.

Analysis of the results of methylation, periodate oxidation and Smith degradation, partial hydrolysis together with the results of NMR studies gives a predicted structure for the polysaccharide ESPS-1 (Fig. 5).

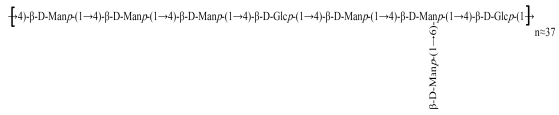

Fig. 5. Predicted structure of ESPS-1.

REFERENCES

[1] W. Ni, X. Zhang, H. Bi, J. Iteku, L. Ji et al., "Preparation of a glucan from the roots of Rubus crataegifolius Bge. and its immunological activity," Carbohydrate research, vol. 344, no. 18, pp. 2512-2518, 2009.

[2] Y. L. Wu, and D. N. Wang, "Structural Characterization and DPPH Radical Scavenging Activity of an Arabinoglucogalactan from Panax notoginseng Root," Journal of natural products, vol. 71, pp. 241-245, 2008.

[3] V. Mozaffarian, A Dictionary of Iranian Plant Names, Sixth ed., p.^pp. 208-210, Tehran: Farhang Moaser, 2008.

[4] M. Dashti, H. Tavakoli, H. ZarifKetabi, and A. Paryab, "Ecological requirements of plant of Eremurus (Eremurusspectabilis M.B.) in Khorasan province," Iranian Journal of Range and Desert Research, vol. 12, no. 2, pp. 153–165 2005.

[5] A. Berdikeev, D. A. Rakhimov, N. V. Plekhanova, and E. S. Kondratenko, "Glucomannan of the tuberous roots of Eremurus cristatus," Chemistry of Natural Compounds, vol. 18, no. 2, pp. 226-227, 1982.

[6] A. Dzhumamuratova, D. A. Rakhimov, and Z. F. Ismailov, "Polysaccharides of Eremurus. X. Characteristics of the polysaccharides of Eremurus lactiflorus and E. luteus," Chemistry of Natural Compounds, vol. 15, no. 5, pp. 526-529, 1979.

[7] A. Dzhumamuratova, D. A. Rakhimov, and E. S. Kondratenko, "Polysaccharides of Eremurus. XV. Structure of the glucomannan of Eremurus lactiflorus," Chemistry of Natural Compounds, vol. 18, no. 6, pp. 642-646, 1982.

[8] A. Dzhumamuratova, D. A. Rakhimov, S. S. Shashkov, and E. S. Kondratenko, "Polysaccharides of Eremurus. XIII. Study of the structure of the partially acetylated glucomannans by the ^{13}C NMR method," Chemistry of Natural Compounds, vol. 18, no. 1, pp. 12-15, 1982.

[9] O. G. Gudyushkina, D. A. Rakhimov, and Z. F. Ismailov, "A study of the polysaccharides of Eremurus robustus," Chemistry of Natural Compounds, vol. 12, no. 5, pp. 582-582, 1976.

[10] M. I. Igamberdieva, D. A. Rakhimov, and Z. F. Ismailov, "The structure of the glucomannan from the tuberous roots of Eremurus altaicus," Chemistry of Natural Compounds, vol. 13, no. 2, pp. 162-167, 1977.

[11] M. I. Igamberdieva, Z. A. Rakhimov, and Z. F. Ismailov, "The structure of the glucomannan from Eremurua altaicus," Chemistry of Natural Compounds, vol. 12, no. 1, pp. 70-71, 1976.

[12] D. A. Rakhimov, M. I. Igamberdieva, K. A. Arifkhodzhaev, and Z. F. Ismailov, "Polysaccharides of Eremurus," Chemistry of Natural Compounds, vol. 10, no. 4, pp. 512-513, 1974.

[13] D. A. Rakhimov, and Z. F. Ismailov, "Polysaccharides of Eremurus. XIV. Structure of the glucomannan from E. turkestanicus," Chemistry of Natural Compounds, vol. 18, no. 1, pp. 127-128, 1982.

[14] M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, "Colorimetric Method for Determination of Sugars and Related Substances," Analytical Chemistry, vol. 28, no. 3, pp. 350-356, 1956.

[15] M. M. Bradford, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the

principle of protein-dye binding," Analytical biochemistry, vol. 72, no. 1-2, pp. 248-254, 1976.
[16] K. Jahanbin, A. R. Gohari, S. Moini, Z. Emam-Djomeh, and P. Masi, "Isolation, structural characterization and antioxidant activity of a new water-soluble polysaccharide from Acanthophyllum bracteatum roots," International Journal of Biological Macromolecules, vol. 49, no. 4, pp. 567-572, 2011.
[17] A. M. Staub, "Removal of protein – Sevag method," Methods in Carbohydrate Chemistry, 5: Academic Press, 1965.
[18] G. Zhao, J. Kan, Z. Li, and Z. Chen, "Structural features and immunological activity of a polysaccharide from Dioscorea opposita Thunb roots," Carbohydrate Polymers, vol. 61, no. 2, pp. 125-131, 2005.
[19] M. M. S. Asker, and B. T. Shawky, "Structural characterization and antioxidant activity of an extracellular polysaccharide isolated from Brevibacterium otitidis BTS 44," Food Chemistry, vol. 123, no. 2, pp. 315-320, 2010.
[20] G. Aspinall, and R. Ferrier, "A spectrophotometric method for the determination of periodate consumed during the oxidation of carbohydrates," Chemical Industry (London), vol. 7, pp. 1216-1221, 1957.
[21] P. W. Needs, and R. R. Selvendran, "Avoiding oxidative degradation during sodium hydroxide/methyl iodide-mediated carbohydrate methylation in dimethyl sulfoxide," Carbohydrate research, vol. 245, pp. 1-10, 1993.
[22] M. T. Dueñas-Chasco, M. A. Rodríguez-Carvajal, P. T. Mateo, G. Franco-Rodríguez, J. L. Espartero et al., "Structural analysis of the exopolysaccharide produced by Pediococcus damnosus," Carbohydrate research, vol. 303, no. 4, pp. 453-458, 1997.
[23] B.-L. Xue, J.-L. Wen, F. Xu, and R.-C. Sun, "Structural characterization of hemicelluloses fractionated by graded ethanol precipitation from Pinus yunnanensis," Carbohydrate Research, vol. 352, no. 0, pp. 159-165, 2012.
[24] N. Smirnova, N. Mestechkina, and V. Shcherbukhin, "The Structure and Characteristics of Glucomannans from Eremurus iae and E. zangezuricus: Assignment of Acetyl Group Localization in Macromolecules," Applied Biochemistry and Microbiology, vol. 37, no. 3, pp. 287-291, 2001.
[25] T. Usui, T. Mizuno, K. Kato, M. Tomoda, and G. Miyajima, "^{13}C NMR Spectra of Gluco-mamno-oligosaccharides and Structurally Related Glucomannan," Agricultural and Biological Chemistry, vol. 43, no. 4, pp. 863-865, 1979.

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