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Isolation and Characterization of Water Soluble Polysaccharides from *Isaria cicadae*

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Abstract: *Isaria cicadae* Miquel is an important medicinal food fungus. The crude polysaccharides from *I. cicadae* were shown to significantly extend the lifespan of fruit flies in our previous study. To understand the underlying mechanism, the composition and structural characterization of polysaccharides from *I. cicadae* were determined. A water-soluble crude polysaccharide, obtained from a cultured *Isaria cicadae* by boiling-water extraction and ethanol precipitation, was fractionated by DEAE-Sepharose column chromatography. The isolate gave four polysaccharide fractions termed B-I-1, B-I-2, B-II-1 and B-II-2. The average ratio of Glu: Man: Gla in B-I-2 was 1.28:1.25:1, B-II-1 was 16.08:1:1.95, and B-II-2, was 1:2.43:2.61. B-I-1 was composed of Ara, Xyl and Glu in the ratio of 1:2.36:33.14. GC and FTIR spectra analysis combined with NMR spectroscopic analysis gave preliminary structures of the four polysaccharide fractions, which may provide an elucidation on the action mechanisms of - polysaccharide on fruit flies proliferation.

Keywords: *Isaria cicadae*, Polysaccharides, GC, FTIR Spectra, NMR Spectra

1. Introduction

Isaria cicadae Miquel, *(Cordycipitaceae*, *Hypocreales*, *Sordariomycetes*, *Pezizomycotina*, *Ascomycota*) [1], is one of the most valued medicinal fungi in Chinese Traditional Medicine. It is commonly known as "Jin Chanhua" (*Cicada* flower) but has also been called as "*Cordyceps* sobolifera" and "Yan flower" in many medical books in China, such as *Chinese Dictionary*, *Xinhua materia medica* and *Sichuan traditional Chinese Medicine*.

As a valued Chinese herb and tonic, *I. cicadae* has a long history of use and a high reputation of value. One of the earliest Chinese accounts of the use of "*Isaria cicadae*" was published in *Leigong Treatise on the Preparation* (fifth century), and later appeared in other famous ancient medical books, such as *Theory of Drug Property* and *Compendium of Materia Medica* (1590). *I. cicadae* has also been shown in recent studies to have multiple pharmacological effects, including immunity regulation [2-4], antitumor activity [5-7], neuroprotective and renoprotective benefits [8-9].

In a previous study, we found that crude polysaccharides from *I. cicadae* extended the lifespan of female and male fruit flies by 18.78% and 26.23% respectively. *I. cicadae* enhanced the activity of SOD and reduced the MDA content in fruit flies [10]. In this study, pure polysaccharide was isolated from *I. cicadae* based on a bioassay-guided fractionation procedure, and structure of the polysaccharide was determined in order to understand the mechanisms of action of polysaccharide on fruit flies proliferation.

2. Material and Methods

2.1. Materials

Powder of *Isaria cicadae* was provided by Zhejiang BioAsia Bio-Pharmaceutical Co., Ltd (China).

2.2. Chemicals and Reagents

T-series dextrans (T-10, T-40, T-70, T-500, T-2000), Dextran, Sephadex G-150, blue dextran-2000 were purchased from Pharmacia (Pharmacia LKB Biotechnology AB, Uppsala,

Sweden); DEAE-cellulose (DE-52) was bought from Whatman (Maidstone, England).

The chemicals and reagents used in this study were of analytical grade, including standard sugars (Arabinose, Rhamnose, Galactose, Mannose, and Xylose), Glucose, sulfuric acid, phenol, sodium periodate, three fluorine acetic acid, sodium borohydride, anhydrous acetic anhydride, sodium hydroxide and DMSO (purchased from Shanghai Zhenqi Bioengineering Institute, Shanghai, China).

2.3. General Methods

Optical rotation was measured with a SpectraMax M2 polarimeter (Molecular Devices, USA). Gas chromatography (GC) was used for identification and quantification. GC was performed on a GC7890A-MSD5975C instrument (Agilent, USA) equipped with HP-5 MS column (30 m × 0.25 mm × 0.25 μm). The column temperature was kept at 80°C for 1 min and then first increased to 200°C at a rate of 5°C /min, then to 215°C at a rate of 2°C/min, finally reaching to 270°C at a rate of 20°C /min. Infrared (IR) spectroscopy of the samples was recorded on a Nicolet 8700 in a range of 400–4000 cm^{-1}. Gel filtration chromatography was carried out on columns of DE-52 and Sephadex G-150 (Whatman, UK) using 0.5 M NaCl. The column was calibrated with T-series dextrans of known molecular weights. All gel filtration chromatography was monitored with phenol– H_2SO_4 method [11].

2.4. Isolation and Purification of Polysaccharides from I. Cicadae

The powder of *I. cicadae* (1.0 g) was extracted with 20 ml of distilled water at 90°C for 2 h, and repeated twice. After vacuum filtration, the aqueous extracts were combined and concentrated to one-fourth of the total volume in vacuum. The extracts were then precipitated in cold 95% (v/v) ethanol to a 75% (v/v) final concentration and incubated at 4°C for a minimum of 24 h. After centrifugation at 5000 rpm for 15 min at 4°C, ethanol was removed and the polysaccharide fraction was redissolved in distilled water. The polysaccharide solution was then treated with proteinase and kept in hot water (65°C) for 1.5 h. Seavg reagent (2-fold of polysaccharide solution) was then added into the solution followed by 30 min ultrasonic. The sample was centrifuged at 8000 rpm for 10 min at 4 °C. The supernatant was retreated in this manner at least 5 times to remove the protein fraction. The resulting aqueous fraction was precipitated again by adding threefold volume of ethanol. After centrifugation, the precipitate was washed with anhydrous ethanol and then dissolved in water and lyophilized (FreeZone12, LABCONCO, USA) to yield the crude polysaccharide corresponding to polysaccharide of *I. cacide* in the subsequent description.

The crude polysaccharide (300 mg) was dissolved in distilled water (10 ml) and the solution was centrifuged at 10000 rpm for 15 min. The supernatant was injected to DEAE-52 column (2.6 cm × 40 cm, Whatman, UK) and eluted with different concentrations of NaCl aqueous solution (0-1 mol/ml) stepwise at a flow rate of 1 ml/ min. Each fraction of 5 ml was collected and monitored by the phenol-sulfuric acid method at 490 nm. The elution profile detected by the phenol-sulfuric acid assay showed two big elution peaks namely as B-I and B-II, respectively. These two fractions were then purified by gel filtration chromatography on a Sephadex G-150 column（2.6 cm × 40 cm, Pharmacia, USA）and eluted with distilled water. Each fraction of 4 ml was collected at a flow rate of 0.45 ml/min and monitored by the phenol-sulfuric acid method at 490 nm using a microplate reader Spectra Max 20 (Molecular Devices, USA).

2.5. Spectrum Evaluation (FTIR Spectra)

IR spectra of the films were recorded with a Nexus-870 Spectrometer (Nicolet, USA), using the photoacoustic technique. KBr pellets containing 1% of the samples were used only to obtain the pure polysaccharide spectra.

2.6. Monosaccharide Composition and Properties

Monosaccharide composition analysis followed the method of Yu et al. (2009) [12]. A polysaccharide fraction of 5 mg was hydrolyzed with 4 ml 2M aqueous trifluoroacetic acid in a sealed tube at 110°C for 4 h. The hydrolysate was concentrated with a rotary evaporator under reduced pressure, and remaining trifluoracetic acid was removed by co-evaporating with methanol (3× 5 ml). The concentrated hydrolysate was dried over P_2O_5 in vacuum overnight. The dried hydrolysate was dissolved in 4 ml acetic acid and stirred at 100°C for 1 h. Excess acetic acid was removed by co-evaporating with toluene (3× 5 ml). The mixture was then concentrated and dried under reduced pressure. The dried residue was dissolved in 3 ml chloroform and then a minimal amount of distilled water was added. This mixture was stirred and then separated with separating funnel. Chloroform layer (10 ml) was for gas chromatography-mass spectrometry (GC-MS) analysis with a GC analyzer GC7890A-MSD5975C (Agilent, USA). The GC column was HP-5 MS with dimension of 30 m × 0.25 mm. The injector and column temperature was 250°C.

2.7. Nuclear Magnetic Resonance (NMR) Spectroscopy

The polysaccharide was dissolved in D_2O at a concentration of approximately 40 mg/mL and deuterium-exchanged 3 times by freeze drying in D_2O. Both ^1H and ^{13}C NMR spectras were recorded on a 600 MHz Bruker INOVA 600NB NMR spectrometer (Brucker, Rheinstetten, Germany). The double quantum filtered correlated spectroscopy (DQF-COSY), heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple-bond correlation (HMBC) experiments were conducted at 65°C.

2.8. Statistical Analysis

These data were presented as means ± SD of three replicates. Statistical analysis was performed using one way analysis of variance (ANOVA). Multiple comparisons of means were done by the least significance difference test. All computations were done by employing the statistical software SPSS (version 18.0).

3. Results and Discussion

3.1. Isolation, Purification and Composition of Polysaccharides from I. Cicadae

The crude polysaccharide was isolated from the hot-water extract of *I. cicadae*. After fractionation on DEAE-cellulose (DE-52) column, B-I (70.86%) and B-II (29.14%) were obtained from the NaCl eluate. The two fractions were purified by gel chromatography on a Sephadex G-150 column, respectively, and showed two symmetrical peak in both B-I and B-II fractions. B-I-1 and B-I-2 were separated from the B-I fraction whileB-II-1 and B-II-2 were obtained from the B-II fraction. The four fractions were repurified on Sphadex G-150 column, respectively, and showed a single peak indicating no other polysaccharide was present in the sample. We concluded that B-I-1 and B-II-1 were homogeneous and the same as B-I-2 and B-II-2 by the following tests. They were all eluted as a single peak from gel-filtration chromatography on a Sephadex G-150 column which was equilibrated in 0.9% sodium chloride. The average molecular weight of B-I-1 and B-II-1 were 108.70 and 114.40 KDa on Sephadex G-150, and B-I-2 and B-II-2 were 43.75 and 49.46 KDa.

The average ratio of Glu: Man: Gla in B-I-2 was 1.28:1.25:1, of B-II-1, 16.08:1:1.95, and of B-II-2, 1:2.43:2.61, while B-I-1 was constituted by Ara, Xyl and Glu in the ratio of 1:2.36:33.14 (Table 1, Figure 1). Mannose and galactose accounted a large ratio in B-I-2 and B-II-2 fractions, which was consistent with reports on polysaccharides from natural *C. cicadae* [13-14]. This indicates that the compositions in polysaccharides are similar between cultured *I. cicadae* and natural *C. cicadae*.

Table 1. *Components of monosaccharides and properties of fractions from Isaria cicadae.*

Samples	B-I-1	B-I-2	B-II-1	B-II-2
Average molecular weights (KDa)	108.70	43.75	114.40	49.46
Sugar component (mol)				
D-Ara	1			
D-Xyl	2.36			
D-Glu	33.14	1.28	16.08	1
D-Man		1.25	1	2.43
D-Gal		1	1.95	2.61

D-Ara, *D*-arabinose; *D*-Xyl, *D*-xylose; *D*-Glu, *D*-glucose; *D*-Man, *D*-mannose; *D*-Gal, *D*-galactose.

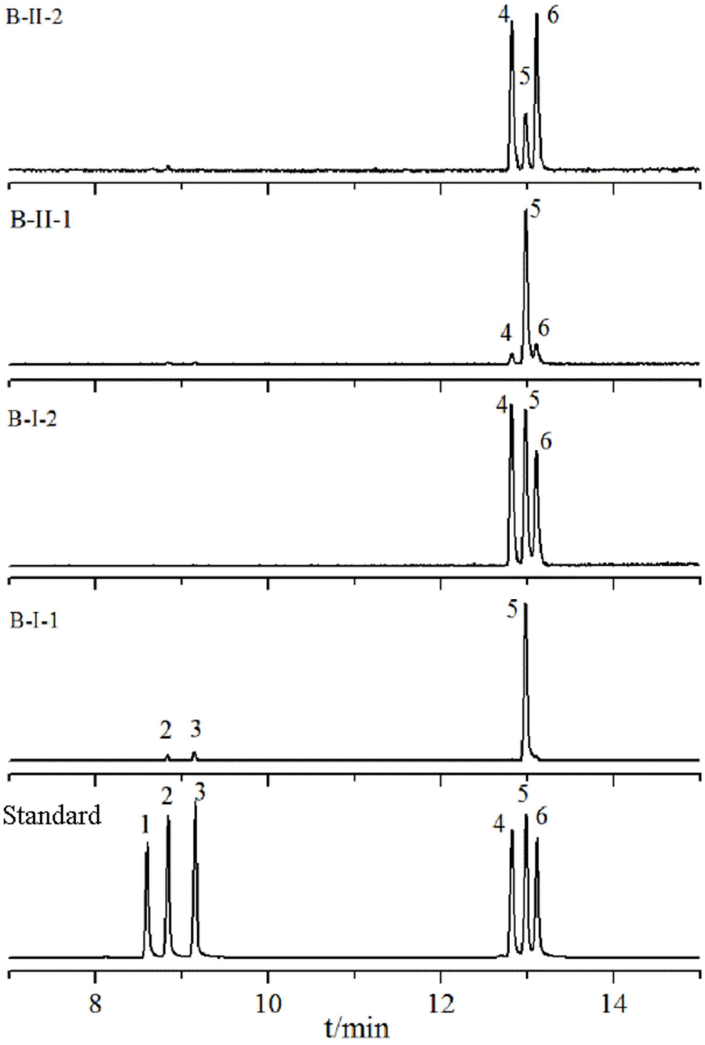

Figure 1. *GC spectrum of monosaccharide references and 4 polysaccharide fractions. 1, L-rhamnose; 2, D-arabinose; 3, D-xylose; 4, D-mannose; 5, D-glucose; 6, D-galactose.*

3.2. FTIR Spectra of Four Polysaccharide Fractions

FTIR spectra of B-I-1, B-I-2, B-II-1 and B-II-2 fractions were drawn and the maximum of the absorption band due to hydroxyl groups (4000-400 cm^{-1}) were measured (Figure 2). The hydroxyl groups self-associated by hydrogen bonds give a strong and broad band, with the center of gravity within 3500-3200 cm^{-1} in all the four polysaccharide fractions. However, it was difficult to find a reasonable correlation between these intense OH stretching modes and vibrational state, due to the complicated network of inter- and intra-molecular hydrogen bonds occurring in the polysaccharide structure.

A second group of bands with moderate intensity in IR spectra was found within 3000-1800 cm^{-1}. These were due to the CH and CH_2 symmetrical and anti-symmetrical stretching vibrations [15]. Another distinct band, which was recorded at about 1646 cm^{-1}, was attributed to C=O stretching, and appeared related to the vibrations of bound water molecules [16]. One band at 1540 cm^{-1} was affected by N-H vibrations, indicating some minimum amount protein was still bound with the polysaccharides in B-II-1 and B-II-2 fractions.

Several bands observed in the range 1450-1220 cm^{-1} overlapped with the CH and CH_2 in-plane bending and wagging vibrations, and to the C-OH deformation modes. Between 1200-1000 cm^{-1} bands were influenced to various extents by other vibrations, such as –OH stretching and anomeric skeleton. Heyn (1974) reported that glycogen exhibited two distinct and well resolved infrared bands in the region 1050 to 970 cm^{-1} [17], while starch showed only one broad band. Galat (1980) believed that the spectral region within 1050 and 970 cm^{-1} could be served as an approximate criterion of polysaccharide heterogeneity [18]. In our study, four fractions exhibited one band of considerable intensity at about 1050 to 1020 cm^{-1}.

The bands detected at about 930 and 761 cm^{-1} in the infrared spectra of B-I-1 fraction, and within 1151 and 761 cm^{-1} in B-II-1 fraction were assigned to out-of-phase C-O-C ring vibrations. This was partly coincide with Kacurakova et al. (2000) [19].

One intensive IR band at about 850 cm^{-1} corresponded to the "α-configuration" bending modes was found in B-I-1 and B-II-1 fractions, while a small band positioned at about 800 cm^{-1} was visible in B-I-2 and B-II-2 fractions, attributed to out-of-plane CH bending deformation vibrations.

Since the four polysaccharides had different chemical structures, it was difficult to find an IR band attributed only to *I. cicadae* polysaccharide groups. However, accumulation of more extensive data on the IR spectra of different sugars and polysaccharides would facilitate estimation of the structure and conformation of polysaccharide structure with the use of computer-assisted analysis.

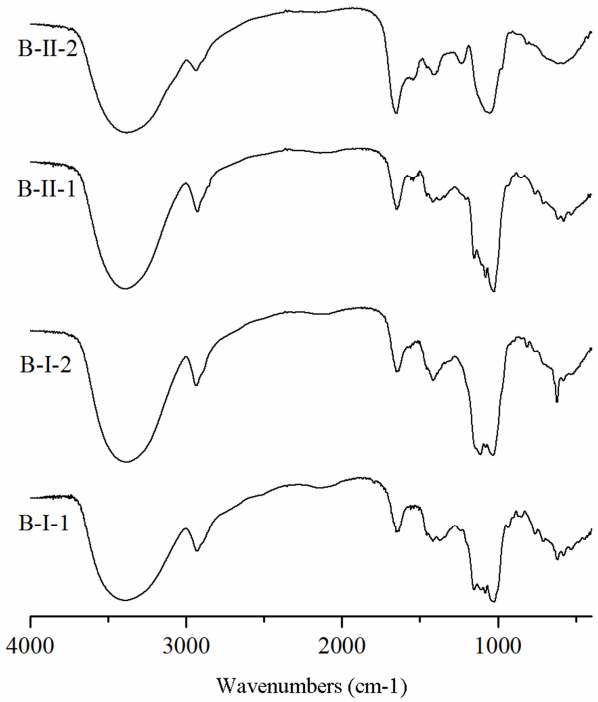

Figure 2. *The FTIR spectra of four polysaccharide fractions from I. cicadae.*

3.3. 1D and 2D NMR Analysis

Signals of four polysaccharide fractions from *I. cicadae* in 1D ^{1}H and ^{13}C NMR and 2D NMR (HSQC, COSY and HMBC) spectra were assigned as completely as possible, based on monosaccharide analysis, linkage analysis and chemical shifts reported in the literature.

Table 2. *Chemical shifts for the resonances of glycosyl residues of B-I-1 in 1D and 2D NMR spectra.*

Pos	Chemical shifts, δ (ppm)				
	δ_H	δ_C	HSQC	^{1}H-^{1}H COSY	HMBC
1	5.34	101.02	1→		H1→C4 H4→C1
2	3.60	72.84			
3	4.10 3.90	85.43 73.91-74.60	3→		
4	3.91-4.10	76.68-82.24	4→	H4→H5	H1→C4 H4→C1 H4→C5
5	3.82	72.83		H5→H4 H5→H6	
6	3.62-3.95	62.65 63.55 62.07 61.77		H6→H5	

With respect to B-I-1, the anomeric proton signals δ 5.34 ppm corresponded to the H-1 of Rhap. The corresponding signal at δ < 102 ppm in ^{13}C NMR was found. So it could be deduced that the residue is attributed to pyran ring with alpha model connecting way of glycosidic bond, which was consistent with the ^{1}H NMR speculation. Based on the data (Table 2) the repeated unit structure was assigned as →4)-α-D-Glc (1→4)-α-D-Glc (1→ (Figure 3).

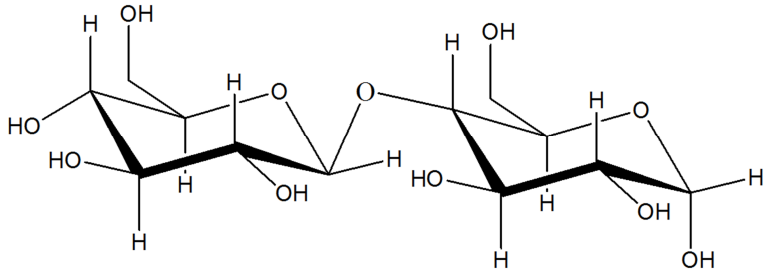

Figure 3. *Structure of B-I-1.*

For B-I-2, the anomeric proton signals δ 5.34，5.14，5.10，4.92 ppm were detected, which was attributed to β or α glycosidic bond. The complete ^{1}H and ^{13}C NMR signal assignments for B-I-2 were summarized in Table 3. From this we deduced that the structure of B-I-2 may be repeated with →1)-α-D-Glc (4→1)-α-Glc (3→1)-α-D-Gal (4→1)-α-D-Gal (3→1)-α-D-Glc (4→, within chain link monose 1 and 4 (Figure 4).

Table 3. *Chemical shifts for the resonances of glycosyl residues of B-I-2 in 1D and 2D NMR spectra.*

Pos	Chemical shifts, δ (ppm)				
	δ_H	δ_C	HSQC	^{1}H-^{1}H COSY	HMBC
Glc 1	5.34 5.14	100.80 101.68 102.03	1→	H1→H4 H1→H2	C1→H4 C1→(Man) H3 H1→(Gal) C3
2	3.34-3.61	70.38-73.19		H1→H2 H2→H3	H2→C3
3	3.75-3.80	82.66-82.72	3→	H3→H4 H3→(Gal) H4	H3→(Gal) C1 H3→C4 C3→(Gal) H1 C3→(Gal) H4
4	3.48-3.63	76.67-78.32	4→	H4→H5 H4→H3	C1→H4 H3→C4 H4→C5
5	3.34-3.61	70.38-73.19		H5→H4 H5→H6	C5→H6 H5→C6 C5→H4
6	3.43-4.25 3.83-4.13	61.48-34.25 66.59-67.69		H6→H5	H6→C5 C6→H5
Gal 1	5.10	105.32 107.16	1→	H1→(Man) H3	C3→(Glc) H1 H1→(Glc) C3 C1→(Man) H3 H1→C4
2	3.34-3.61	70.38-73.19		H1→H2 H2→H3	H2→C3
3	3.93-4.05	80.16-83.09	3→	H2→H3	C3→(Glc) H1
4	4.08-4.22	74.68-78.74	4→	H4→H5 H4→(Glc) H3	C4→H1 C4→(Man) H1
5	3.34-3.61	70.38-73.19		H5→H4 H5→H6	C5→H6 H5→C6 C5→H4
6	3.43-4.25 3.83-4.13	61.48-34.25 66.59-67.69		H6→H5	H6→C5 C6→H5
Man 1	4.92	98.75 99.11	1→		H1→(Glc) C3 H1→(Gal) C4 H1→C2
2	3.71-3.92	71.57-74.53		H2→H3	C2→H1
3	4.12-4.16	87.96-88.52	3→	H3→(Gal) H1	H3→(Gal) C1 H3→(Glc) C1
4	3.70	70.32			
5	3.71-3.92	71.57-74.53		H5→H6	H5→C6
6	3.43-4.25 3.83-4.13	61.48-34.25 66.59-67.69		H5→H6	C6→H5

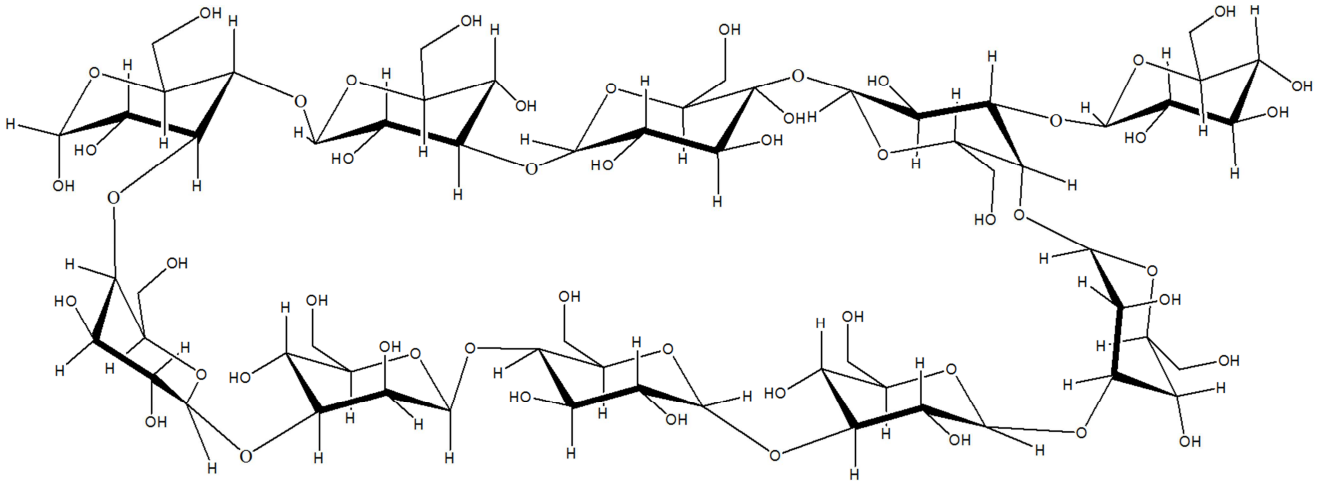

Figure 4. *Structure of B-I-2.*

For B-II-1, the data showed that it was very similar to B-I-1. The anomeric proton signals δ 5.34，5.14，5.10，4.92 ppm were detected, which were attributed to α glycosidic bond, and in the 13C spectrum, the δ < 102 ppm was attributed to pyran ring with alpha model connecting way of glycosidic bond, which was consistent with the ^{1}H NMR speculation (Table 4). The data showed that the repeating unit of B-II-1 was →1)-α-D-Glc (4→1)-α-D-Glc (4→1)-α-D-Glc (4→1)-α-D-Glc (4→1)-α-D-Glc (4→1)-α-D-Glc (4→1)-α-D-Glc (4→1)-α-D-Glc (4→, with the C3 in the fourth Glc was replaced by α-D-Glc (3→4)-α-D-Gal (Figure 5).

Table 4. *Chemical shifts for the resonances of glycosyl residues of B-II-1 in 1D and 2D NMR spectra.*

Pos	**Chemical shifts, δ (ppm)**				
	δ_H	δ_C	**HSQC**	**^{1}H-^{1}H COSY**	**HMBC**
Glc 1	5.34 5.16 5.10	101.75 101.15 100.88	1→		H1→C4
2	3.60-3.81	71.82-73.29			
3	3.74-4.12 4.19	82.40-82.91 87.90	3→	H3→H4	C3→(Gal) H4 H3→C4
4	3.93-4.22	75.57-78.63	4→	H4→H5	H3→C4 H1→C4
5	3.60-3.81	71.82-73.29		H5→H6 H5→H4	H6→C5
6	3.60-4.29	61.54-63.82 66.54-67.84	6→	H5→H6	H6→C5
Gal 1	4.92	99.12	1→		H1→C2
2	3.60-3.81	71.82-73.29			H1→C2
3	4.06-4.12	82.57-82.91	3→		(Glc) C3→H4
4	3.39	70.88		H4→H5	
5	3.60-3.81	71.82-73.29		H4→H5	
6	3.67-4.29	61.54-63.82			

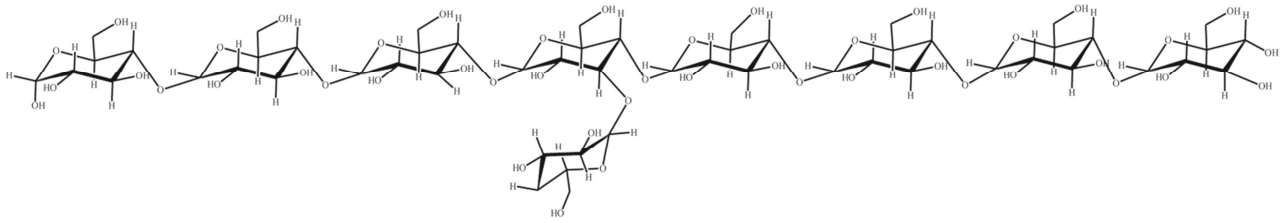

Figure 5. *Structure of B-II-1.*

For B-II-2, based on 1H and 13C NMR spectrum and the data in Table 5, we concluded that the main chain was →1)-α-D-Gal (4→1)-α-D-Gal (3→1)-α-D-man (3→1)-α-D-man (3→, with the second Gal replaced by α-D-Gal (4→1)-α-D-Glc (Figure 6).

Table 5. *Chemical shifts for the resonances of glycosyl residues of B-II-2 in 1D and 2D NMR spectra.*

Pos	Chemical shifts, δ (ppm)				
	δ_H	δ_C	HSQC	^{1}H-^{1}H COSY	HMBC
Gal 1	5.10 5.08	101.74 107.34	1→		H1→C4
2	3.63-3.96	70.91-72.54			
3	3.74-4.18	82.75-83.03	3→	H3→H4	H4→C3 C3→H6 C3→(Man) H1
4	3.85-4.22	74.60-78.84	4→	H4→H5 H4→H3	C4→H5 C4→H1
5	3.63-3.96	70.91-72.54		H5→H4 H5→H6	H5→C4
6	3.54-4.24	60.53-63.82 63.82-67.66	6→	H6→H5	
Man 1	5.15 5.18	100.68 104.44	1→	H1→H3	H1→(Gal) C3 H1→C3
2	3.63-3.96	70.91-72.54			
3	4.12	87.92	3→	H3→H1	C3→H1
4	4.04	70.91			
5	3.63-3.96	70.91-72.54			
6	3.54-4.24	60.53-63.82 63.82-67.66	6→		
Glc 1	5.34	105.30	1→		
2	3.63-3.96	70.91-72.54			
3	4.04	73.47			
4	3.66	77.30	4→		
5	3.63-3.96	70.91-72.54			
6	3.54-4.24	60.53-63.82 63.82-67.66	6→		

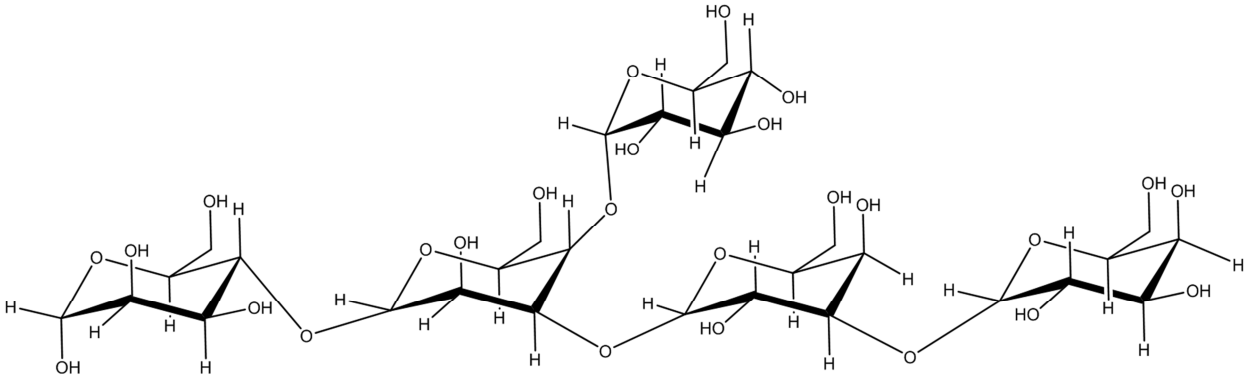

Figure 6. *Structure of B-II-1.*

4. Conclusion

According to the above results, it was concluded that the water extract of *Isaria cicadae* predominantly contained four types of water extractable polysaccharides (B-I-1, B-I-2, B-II-1 and B-II-2). According to their FTIR spectra, it is possible to assume that the four polysaccharide fractions exhibited various extents marker vibrations of polysaccharide substance, such as O-H, C-H, C=O and –OH stretching. However, special bands were also found in the IR spectra of each fraction, making it not feasible to use some vibrations as marker bands for *I. cicadae* polysaccharides. Every polysaccharide fraction was constituted by *D*-glucose, *D*-mannose and *D*-galactose in different ratios except for B-I-1, which was constituted by *D*-arabinose, *D*-xylose and *D*-glucose. Moreover, preliminary structure of four polysaccharide fractions from *I. cicadae* were proposed as follows: →4)-α-D-Glc (1→4)-α-D-Glc (1→ for B-I-1; →1)-α-D-Glc (4→1)-α-Glc (3→1)-α-D-Gal (4→1)-α-D-Gal (3→1)-α-D-Glc (4→, within chain link monose 1 and 4 for B-I-2; →1)-α-D-Glc (4→1)-α-D-Glc (4→1) -α-D-Glc (4→1)-α-D-Glc (4→1)-α-D-Glc (4→1)-α-D-Glc (4→1)-α-D-Glc (4→1)-α-D-Glc (4→, with the C3 in the fourth Glc was replaced by α-D-Glc (3→4)-α-D-Gal for B-II-1; →1)-α-D-Gal (4→1)-α-D-Gal (3→1)-α-D-man (3→1)-α-D-man (3→, with the second Gal replaced by α-D-Gal (4→1)-α-D-Glc for B-II-2. Preliminary activity tests showed that *I. cicadae* were effective in prolonging the lifespan of fruit flies and improving antioxidant activity. Further studies are necessary to relate such activities with the pharmacological effect of the four polysaccharide fractions.

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