

Antioxidant activities of polysaccharide fractions isolated from burley tobacco flowers

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Summary

The crude polysaccharide extracted from the flowers of burley tobacco hybrid Eyan 1 was fractionated on a Sepharose CL-6B chromatography to get two purified polysaccharide fractions (termed Fr-I and Fr-II). The monosaccharide compositions revealed both fractions were heteropolysaccharide. Fr-I was mainly composed of mannose, galactose, ribose and arabinose, while Fr-II was composed of mannose, galactose and glucose. The IR spectrum of Fr-I and Fr-II revealed the typical characteristics with β -configuration. Furthermore, TGA (Thermogravimetric analysis) indicated the degradation temperature (Td) of the Fr-I and Fr-II was 210 °C and 185 °C, respectively. Both Fr-I and Fr-II possessed good scavenging abilities on hydroxyl radicals (IC₅₀ 8.26 mg/ml and 6.23 mg/ml, respectively) and DPPH radicals (IC₅₀ 20.3±1.20 µg/ml, 17.5±1.28 µg/ml, respectively). Fr-II showed higher antioxidant properties than Fr-I ($p < 0.05$). In a ¹³C NMR analysis of Fr-II, three anomeric peaks appeared at 102.95, 95.79 and 91.85 ppm, which were assigned to the mannose (C-1), galactose (C-1) and glucose (C-1) residues, respectively.

Keywords: extraction, purification, polysaccharide, antioxidant activity, tobacco flower

Introduction

Water-soluble polysaccharides, long-chain polymers with high molecular weight, are generally present in cell walls of higher plants. In recent years, there has been a growing interest in polysaccharides obtained from higher plants that may have wide diversity of chemical structures and biological activities (Kardošová and Machová, 2006; Srivastava and Kulshreshtha, 1989). The polysaccharides extracted from higher plants are widely used in diverse fields in the food, pharmaceutical and many other industries (Ridley et al., 2001).

The polysaccharides from plants have proved to be one of the most promising groups of antioxidant compounds (Ng et al., 2004). Although many studies have assessed the antioxidant properties of polysaccharide from fruits and vegetables, much less is known about the antioxidant characteristics of polysaccharide from another commonly ingested plant product, tobacco (Nguema-Ona et al., 2012). To the best of our knowledge, although the isolation and characteristics of water-soluble polysaccharide for burley tobacco are investigated, there is no information available about polysaccharide from burley tobacco flowers in literatures (Wang et al., 2012; Wright Jr. et al., 1962).

Ultrasound-assisted extraction (UAE) is a very effective technique for the extraction of bioactive substances from plants. The mechanochemical effect

of ultrasound can accelerate the extraction process due to disruption of cell walls and enhanced mass transfer of the cell contents without changing the structural and molecular properties of plant materials (El'Piner, 1964). Burley tobacco (*Nicotiana tabacum* L., belongs to the family Solanaceae and genus *Nicotiana*), used primarily for cigarette production. The objective of this study is to isolate and purify the polysaccharides of burley tobacco (hybrid Eyan 1) flowers. Purified polysaccharides obtained were analyzed, and their associated antioxidant capabilities were evaluated for the first time.

Materials and methods

Materials and chemicals

The burley tobacco flowers were kindly provided from the Yuxi Tobacco Planting Area (Yunnan, China). A voucher specimen (Eyan 1) has been deposited in our laboratory. The tobacco flowers were dried at 45 °C and sieved using a 40-mesh sieve (pore size 0.42 mm). The standard monosaccharides were obtained from Sigma–Aldrich (St. Louis, MO, USA), whilst Sepharose CL-6B was from the Sigma Chemical Co. (St Louis, MO). All other chemicals were of analytical reagent grade.

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Extraction, isolation and purification of the polysaccharides

The plant material was extracted by the ultrasound-assisted extraction (UAE) method as developed by Wu, Ling and Chau with some modifications (Wu et al., 2001). One gram of dry tobacco flower powder was extracted with distilled water in a JY92-2D Ultrasonic cell grinder (Ningbo Scientz Biotechnology Co., LTD, China). The extraction experiment was carried out according to the optimal conditions (extraction power 300 W, extraction time 6 min, ratio of water to raw material 35:1 ml/g, and extraction temperature 70 °C). The precipitates were washed three times with 50, 75 and 100 % ethanol and filtered in order to remove the mono- and disaccharides. The ethanol precipitate of the crude polysaccharide was deproteinized using Sevag reagent (1:4 n-butanol/chloroform, v/v). After centrifugation, the aqueous phase was dialyzed against deionized water and dried under vacuum freeze-drying to obtain the crude polysaccharide. The crude polysaccharide was re-dissolved in 0.2 M NaCl buffer, and loaded onto a Sepharose CL-6B column (2.4 cm×100 cm) and eluted with the same buffer at a flow rate of 0.6 ml/min. The protein moiety in the polysaccharide was monitored according to the Bradford method (Bradford, 1976). The total carbohydrate content in the polysaccharide was detected by the phenol-sulfuric acid method (Dubois et al., 1956). The procedure of extraction and purification was repeated at least ten times to collect enough amount and the purified fractions were lyophilized for further chemical analysis and antioxidant activity tests.

Monosaccharide composition analysis

For the identification and quantification of monosaccharide, polysaccharide fraction (5 mg) was hydrolyzed with 2 mL of 2 M trifluoroacetic acid (TFA) at 110 °C for 2 h. The hydrolyzate was repeatedly co-concentrated with methanol, reduced with NaBH₄ for 30 min at 20 °C and acetylated with acetic anhydride and pyridine at 100 °C for 20 min. The internal standard sugars were prepared and subjected to GC/MS analysis separately in the same way. The alditol acetates of polysaccharide fraction were analyzed by GC/MS (Varian Co., Model: Star 3600 CX, Lexington, MA, USA) fitted with a fused silica capillary column (Na form, 300mm × 0.25 mm, Supelco Inc., Bellefonte, PA, USA) and a flame ionization detector (Wang et al., 2012).

Fourier transform-infrared (FT-IR) spectroscopy

The FT-IR spectra of the purified fractions (1 mg) were recorded on a Bruker Tensor 27 FT-IR spectrometer. The dried sample was ground with potassium bromide powder and pressed into pellet for spectrometric measurement in the frequency range of 550 to 4,000 cm⁻¹.

Thermo gravimetric analysis

Thermogravimetric analysis of the polysaccharide was done using TA Q5000IR TGA apparatus using 15 mg purified polysaccharide fraction of the test material. The TGA curve plots the TGA signal, converted to percent weight change on the Y-axis against the reference material temperature on the X-axis.

¹H and ¹³C NMR spectroscopy

Nuclear magnetic resonance (NMR) spectra were obtained using a Bruker Avance 400 MHz spectrometer (Bruker Co., Billerica, MA) with a 5 mm inverse probe. Proton spectra were run at a probe temperature of 25 °C, while carbon spectra were obtained at 25 °C. The samples were dissolved in D₂O (99.96 %) at concentrations of 5 mg/mL (for ¹H NMR) and 20 mg/mL (for ¹³C NMR). ¹H chemical shifts were referenced to internal D₂O (4.7 ppm at all temperatures).

Antioxidant activity assays

For the evaluation of antioxidant activity of polysaccharide extracted from tobacco flowers, OH radical scavenging activity and DPPH radical scavenging activity were determined according to the methods of Wang et al. (2012) and Eloff, Katerere and McGaw (2008), respectively. In both assays the polysaccharide samples were predissolved in water and tested at various concentrations in parallel with vitamin C (Vc) as an antioxidant reference (positive control). The radical-scavenging activities of samples, expressed as percentage inhibition of OH and DPPH, were calculated according to the formula: radical scavenging rate (%) = (A_{sample} - A_{blank}) / (A_{control} - A_{blank}) × 100 Where A_{sample}, A_{control}, and A_{blank} were defined as absorbances of the sample, control (without polysaccharide) and blank (without H₂O₂, DPPH), respectively. The experiments were performed in triplicate. Data were analyzed by using SPSS (version 11.0, Chicago, IL). Probability values < 0.05 and < 0.01 were regarded as statistically significant and highly significant, respectively.

Results and discussion

Isolation, purification and analysis of carbohydrates

In gel filtration chromatography of the culture filtrate on Sepharose CL-6B, two fractions (designated as Fr-I and Fr-II) of polysaccharide were obtained with the yield of 2.62 mg (Fr-I) and 6.96 mg (Fr-II) from one gram tobacco powder (Fig. 1). Although further detection by electrophoresis is necessary, it might be revealed that Fr-II was glycoprotein. Two fractions were also observed from Virginia tobacco in our previous publication. However, the different is, Fr-I of Virginia tobacco polysaccharide may be a glycoprotein, whereas Fr-I of burley tobacco polysaccharide is not (Xu et al., 2013). Both of them appeared as a white powder. The detailed monosaccharide compositions of carbohydrate in the two polysaccharide fractions by GC-MS analysis method are illustrated in Table 1. The result indicated

that both fractions are heteropolysaccharide. Fr-I was mainly composed of mannose (35.2 %), galactose (23.6 %), ribose (13.5 %) and arabinose (10.9 %), while Fr-II was composed of mannose (24.3 %), glucose (22.1 %) and galactose (15.2 %).

Table 1. Carbohydrate composition in the purified polysaccharide fractions (Fr-I and Fr-II) extracted from the burley tobacco flowers

Carbohydrate composition (%)	Fr-I	Fr-II
Arabinose	10.9	6.2
Rhamnose	7.7	6.4
Ribose	13.5	7.2
Xylose	1.7	4.3
Talitol	1.9	4.2
Galactose	23.6	15.2
Glucose	5.5	22.1
Mannose	35.2	24.3
Galacturonic acid	0	4.5
Glucuronic acid	0	5.7

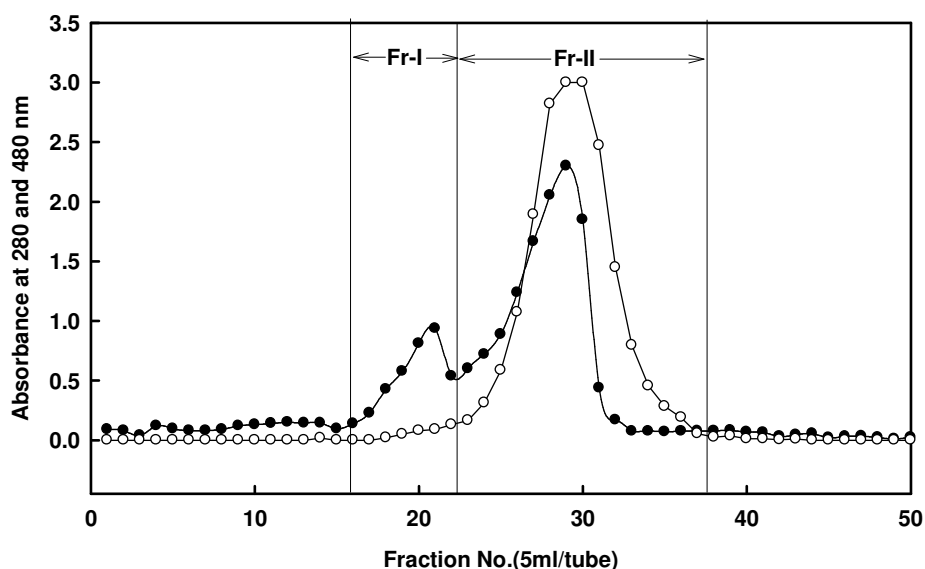


Fig. 1. Elution profiles of the pectic polysaccharide in Sepharose CL-6B chromatography. Elutes were analyzed by measuring the absorbance at 490 nm for carbohydrate (●) and the absorbance at 280 nm for protein (○)

FT-IR spectroscopy

In order to investigate the functional groups of the purified polysaccharides, the spectra were recorded at the absorbance mode from 550 to 4,000 cm^{-1} . As shown in Fig. 2, the IR spectra of Fr-I and Fr-II was displayed an intensity and broad band at around 3300 cm^{-1} , which assigned to the hydroxyl groups stretching vibration. The weak intensity of band attributed to C-H group antisymmetrical stretching vibration (2930 cm^{-1}) could also be observed. The bands around 1605 cm^{-1} suggested the presence of the ester carbonyl

groups (C=O). The band at 1345 cm^{-1} of Fr-II corresponds to a CO-NH deformation and to the CH_2 group (amide III), due to the formation of CO-NH group (Paulino et al., 2006). This result confirmed that Fr-II was glycoprotein, which was coincident with the elution profiles of polysaccharide. The prominent absorption bands at around 1069 cm^{-1} suggested the presences of a pyranose ring (Synytsya et al., 2009). The characteristic absorption at 890 cm^{-1} was found in the IR spectrum, indicated both Fr-I and Fr-II contain β -dominating configuration in pyranose form sugar (Fellah et al., 2009).

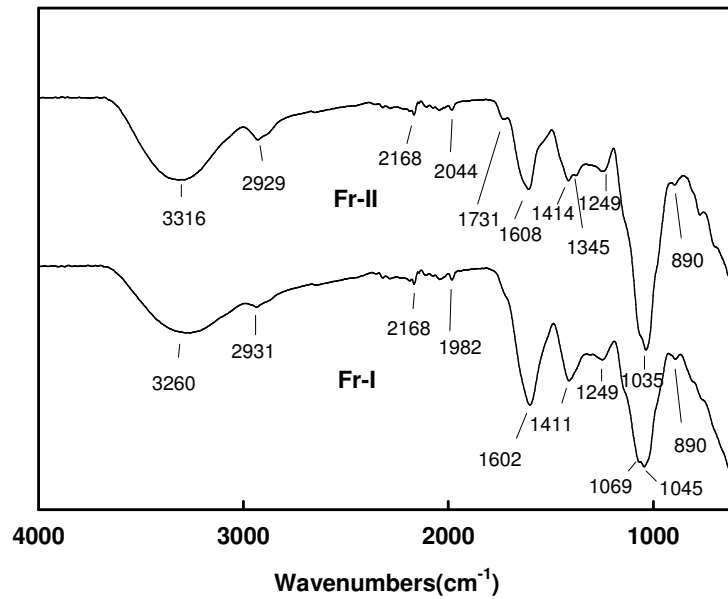


Fig. 2. The FT-IR spectra of the pectic polysaccharide fractions (Fr-I and Fr-II) from burley tobacco flowers

Thermal analysis of polysaccharide

Thermal stability is one of the most important physicochemical properties, which can be determined by thermogravimetric analysis (TGA). The TGA analysis of purified polysaccharide fractions was carried out dynamically (weight loss versus temperature) and the experimental results are presented in Fig. 3. According to the TGA curve of each fraction, the degradation temperature (Td) of Fr-I and Fr-II was

determined as 210 °C and 185 °C, respectively. However, the Td of Fr-I and Fr-II of Virginia tobacco polysaccharide was determined as 185 °C and 190 °C, respectively (Xu et al., 2013). Furthermore, the weight of each fraction was dramatically lost around 260 °C and continued gradually to decrease and the final residue was 13.18 % for Fr-I and 21.25 % for Fr-II. Through this analysis, it appears that the two fractions from burley tobacco flowers possess high thermal stability.

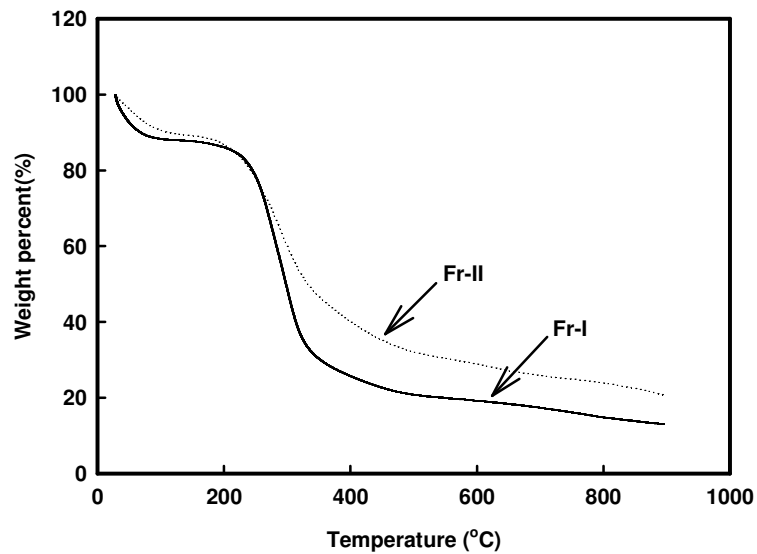


Fig. 3. TGA thermogram of pectic polysaccharide fractions (Fr-I and Fr-II) from burley tobacco flowers

Antioxidant properties analysis

Two biochemical methods (including hydroxyl and DPPH radical scavenging assay) were used to evaluate the antioxidant capacities of two polysaccharide fractions *in vitro*. The effects of hydroxyl radical scavenging activities of Fr-I and Fr-II were given in Fig. 4A. They showed both fractions have the activity of scavenging hydroxyl radicals. The scavenging activities of Fr-I and Fr-II increased with the increase of polysaccharide concentration ranging from 0.5 to 10 mg/ml. The IC₅₀ values of Fr-I and Fr-II in the hydroxyl test system were calculated as 8.26 mg/ml and 6.23 mg/ml, respectively, which were higher than that of positive control Vc (0.49 mg/ml, $p < 0.05$). Fig. 4B describes the scavenging

abilities of Fr-I and Fr-II on DPPH radical. It showed that the scavenging abilities of Fr-II on inhibition of the DPPH radical were related to the concentration of the samples ($p < 0.05$). However, Fr-I exhibited a lower radical scavenging activity than Fr-II at the same concentration point. The higher level of DPPH scavenging ability of Fr-I was not observed in higher concentration range, while the highest scavenging ability of Fr-II was close to that of Vc. The order of IC₅₀ values for DPPH radical was Fr-II (17.5±1.28 µg/ml) > Fr-I (20.3±1.20 µg/ml) > Vc (28.7± 2.60 µg/ml). Compared Fr-I, Fr-II had a higher the scavenging ability, which is related to the different composition and molecular weight between them (Zhao et al., 2006).

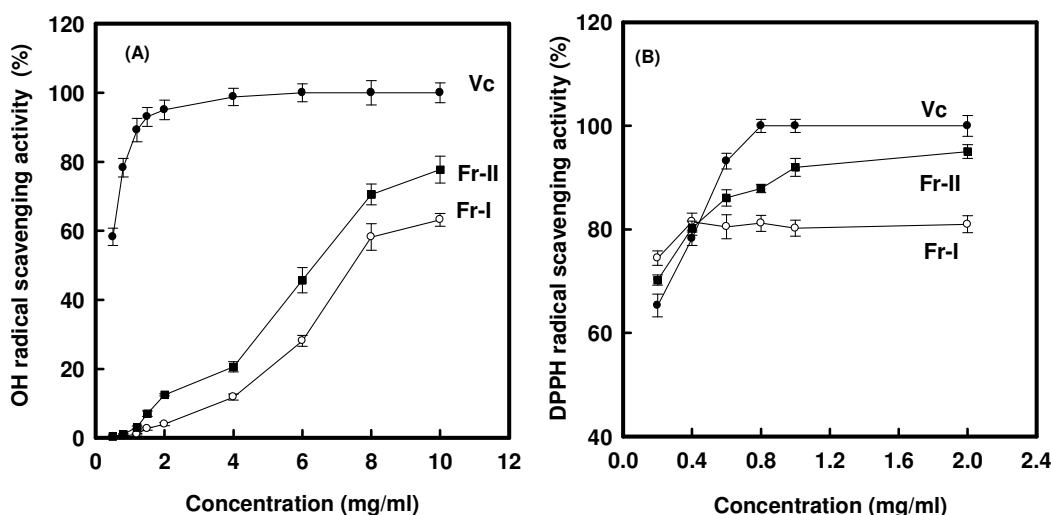


Fig. 4. Antioxidant activity of pectic polysaccharide fractions (Fr-I and Fr-II) from burley tobacco flowers. The results represent mean \pm S.D. ($n = 3$). OH (A) and DPPH (B) radical scavenging activity of polysaccharide. Vitamin C (●), Fr-I (◐) and Fr-II (○)

¹H and ¹³C NMR spectroscopy of Fr-II

Since the antioxidant activity of Fr-II was much higher than Fr-I, resonances of the anomeric protons of Fr-II in ¹H and ¹³C NMR spectra were well separated and identified. From the ¹H NMR spectrum of the anomeric protons (Fig. 5A), three resonances were identified in the region for anomeric protons at δ 4.50-5.50, δ 5.13, and δ 4.96 ppm. This indicated Fr-II was mainly consist of three different types of monosaccharide residues. The strong signal at δ 4.50-5.50 ppm was attributed to α -D-mannose residues (Chai et al., 2005). δ 5.13 and δ 4.96 ppm were assigned to reduced end-chain of α -D-galactose and α -

D-glucose, respectively (Kibalchenko et al., 2010; van Leeuwen et al., 2008). The ¹³C NMR spectra of Fr-II are shown in Fig. 5B. The high resolution ¹³C NMR spectra of the Fr-II contained three main signals in the anomeric region at δ 102.95, 95.79 and 91.85 ppm, which were assigned to the mannose (C-1), galactose (C-1) and glucose (C-1) residues, respectively (Bagno et al., 2007; Gubica et al., 2009; Kibalchenko et al., 2010). According to the NMR results, therefore, the main components of Fr-II were α -D-mannose, α -D-galactose and α -D-glucose, which was coincident with the results of monosaccharide composition analysis of Fr-II.

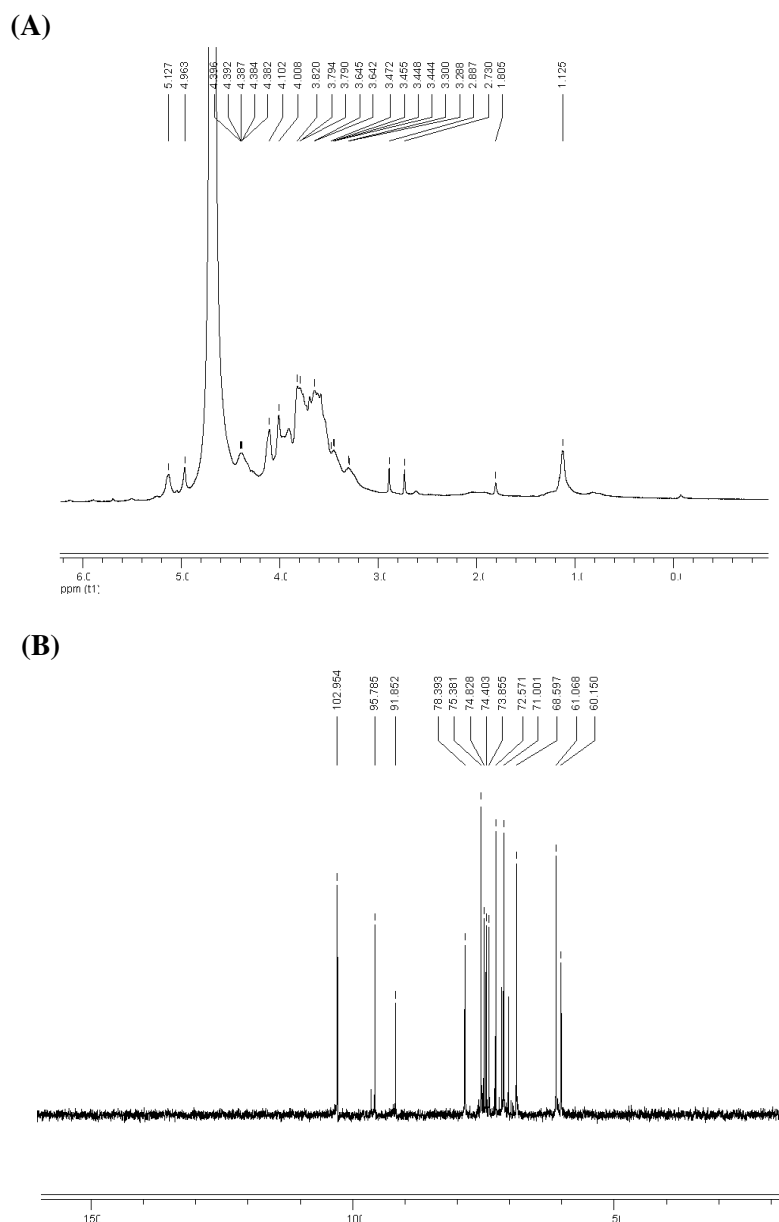


Fig. 5. The $^1\text{H-NMR}$ (A) and $^{13}\text{C-NMR}$ (B) spectra of the purified Fr-II from burley tobacco flowers

Conclusions

In this study, orthogonal matrix method was applied to optimize ultrasound-assisted extraction parameters for polysaccharide from burley tobacco flowers. Two polysaccharide fractions were obtained by Sepharose CL-6B column chromatography. The characterizations of both fractions was analyzed with FT-IR spectroscopy, GC and TGA. The Fr-II was also identified by ^1H and ^{13}C NMR spectroscopy. *In vitro* antioxidant activities studies indicated both Fr-I and Fr-II exhibit strong activity in scavenging free radicals.

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