Ultrasound-Assisted Extraction and Characterization of Pectic Polysaccharide from Oriental Tobacco Leaves

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Summary: Ultrasound-assisted extraction (UAE) of pectic polysaccharide from oriental tobacco leaves was studied by orthogonal matrix method ($L_9(3)^4$). Furthermore, the crude polysaccharide was purified and two components (Fr-I and Fr-II) were obtained. FT-IR spectral analysis of the purified EPS revealed prominent characteristic groups. The monosaccharide composition analysis indicated the main composition between Fr-I and Fr-II was different. Furthermore, thermo gravimetric analysis (TGA) indicated the degradation temperature (Td) of the Fr-I (215 °C) was higher than those of Fr-II (162 °C). Detected by the pyrolysis GC/MS, though the main kinds of pyrolysis products from both Fr-I and Fr-II were similar, the larger amount of heterocycle and aromatic compounds liberated after hydrolysis of Fr-II. Finally, On the basis of the antioxidant activity test *in vitro*, Fr-II has stronger antioxidant activities than Fr-I. The thermal behavior and antioxidant activity might be attributed to the configuration of the sugar units and chemical compositions.

Keywords: Antioxidant activity, Extraction, Optimization, Oriental tobacco leaves, Polysaccharide

Introduction

Pectin, also known as pectic polysaccharide, is one of major group of polysaccharide present in the cell wall of dicotyledonous plants, and has been extensively investigated using chemical analysis and enzymatic degradation [1]. The complicated structure and the retention by plants of large number of genes required to synthesize pectin, suggest that pectin has multiple functions in plant growth and development [2]. Pectic polysaccharide extracted from higher plants is widely used in food, pharmaceutical and cosmetic industries as gelling agents, thickeners, and stabilizing agents.

To the best of our knowledge, although the characteristics of pectic polysaccharide for several plant species are known, polysaccharide from tobacco leaves has been little investigated [3]. The pectic polysaccharide of tobacco vegetative tissues is of interest because tobacco is used as scientific model system to study a number of important agriculturally and industrially relevant processes [4].

Ultrasonic treatment seems to be very promising to obtain pectic polysaccharide from different plant materials in recent years and showed the significant extraction efficiency [5]. The ultrasonically assisted extraction of bioactive principles from herbs has been reviewed [6]. This great extraction efficiency by ultrasonic treatment is mainly attributed to its mechanical effects (including microjetting and microstreaming), which promote targeted compounds to move from the sample matrix into the solvent. However, the mechanical effects do not induce changes in molecular structure [7].

Oriental tobacco is a class of tobacco grown in Turkey, Greece, and neighboring areas. It has strong characteristic flavor, low in nicotine, and high in reducing sugars, acids, and volatile flavor oil. The purpose of this study was to optimize the extraction conditions to simultaneously obtain the pectic polysaccharide from oriental tobacco leaves using orthogonal matrix method. Then, the polysaccharide was further purified by Sepharose CL-6B chromatogram. Gas chromatography/mass spectrometry (GC/MS) and infrared spectrophotometry were applied to identify the chemical composition and structures. Thermal gravimetric analysis (TGA) confirmed the polysaccharides thermal stability. The pyrolysis gas chromatography–mass spectrometry (Py-GC/MS) was used to detect quantitatively pyrolysis products. Finally, the comparative evaluation of the antioxidant capacities of leaf polysaccharide was also conducted in the present study.

Experimental

Materials and Reagents

The leaves of oriental tobacco (*Nicotiana tabacum* L.) were kindly provided from the R&D center of Shanghai Tobacco (Group) Co., Ltd. (Shanghai, China). A voucher specimen (YNOTBS1)

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is deposited at has been deposited with our laboratory. The tobacco leaves was dried at 45 ^{o}C and ground to pass through a 40-mesh sieve prior to experiments. 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 used were of analytical grade.

Extraction of Polysaccharide from Tobacco

The process of polysaccharides extraction from powder of the tobacco leaves by ultrasonic treatment was performed in an ultrasonic cell disintegrator (JY92-II, Ningbo Scientz Biotechnology Co., LTD, Ningbo, Zhejiang Province, China). The procedure of UAE was developed by Wu *et al*. with some modifications [8]. One gram of the tobacco leaves (PTL) powder was extracted with distilled water in a cylindrical probe (6mm diameter). The extractions were carried out referring to conditions in Table-1. Debris fragments of polysaccharide extracts were removed by centrifugation. The solution of polysaccharide was concentrated with a rotary evaporator and precipitated with four volumes of absolute ethanol for 48 h at 4 ^{o}C. The precipitate that formed was collected by centrifugation at 10000 rpm and repeatedly washed sequentially with smaller amounts of ethanol, acetone, and ether, then deproteinized using Sevag reagent (1:4 n-butanol/chloroform, v/v). The aqueous phase was dialyzed against deionized water and lyophilized to obtain the crude polysaccharide (CPD). The extraction yields, subject of this study, were calculated as follows:

$$\text{Extraction yields (\%, w/w)} = W_{CPD}/W_{PTL} \times 100 \quad (1)$$

where W_{CPD} was defined as weight of CPD whereas W_{PTL} was defined as weight of PTL used.

Table-1: Experimental factors and their levels for Orthogonal Projects.[a]

Level	Power (A) W	Time (B) min	Ratio of Solvent to Solid (C)	Temperature (D)^{o}C
1	400	4	20	40
2	500	6	25	50
3	600	8	30	60

[a]Symbols A, B, C, and D represent factors of extraction. Symbols 1, 2, and 3 represent concentration levels of each factor.

Optimization of Polysaccharide Extraction

An orthogonal $L_9(3)^4$ test design was used to investigate the optimal extraction condition of polysaccharide from oriental tobacco leaves. As seen from Table-1, the extraction experiment was carried out with 4 factors and 3 levels, namely ultrasonic power (400, 500, 600W), extraction time (4, 6, 8 min), ratio of water to raw material (20, 25, 30) and extraction temperature (40, 50, 60 ^{o}C). The range of each factor level was based on the results of preliminary experiments. The extraction yield (%) of polysaccharide was the dependent variable. The polysaccharide obtained from the above 9 tests was operated following the method in the section of extraction of polysaccharide from tobacco.

Purification of Polysaccharide

The polysaccharide was re-dissolved in 0.2 M NaCl buffer, and applied to 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. Fractions (5.0 mL/tube) were collected by a fraction collector. The total carbohydrate content in the polysaccharide was determined by the phenol sulfuric acid method, using glucose as standard [9]. The total sugar content in the polysaccharide was determined by phenol sulfuric acid method using glucose as the standard. The peaks with the highest polysaccharide content were collected, dialyzed and then freeze-dried for further analysis.

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 ^{o}C for 2 h. The hydrolyzate was repeatedly co-concentrated with methanol, reduced with $NaBH_4$ for 30 min at 20 ^{o}C and acetylated with acetic anhydride and pyridine at 100 ^{o}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.

Fourier Transform-Infrared (FT-IR) Spectroscopy

FT-IR Spectroscopy (Bruker Tensor 27) was analyzed using the KBr disc for detecting functional groups. The purified polysaccharide fractions (1 mg) were ground with 300 mg KBr powder and then pressed into pellets for transform IR spectral measurement on a Mattson Instrument from 550 to 4,000 cm^{-1}. Spectra were corrected for wave number dependent signal-detection efficiency of the setup using the white light spectrum of a temperature-calibrated tungsten band lamp.

Thermo Gravimetric Analysis and Pyrolysis GC/MS Analysis

Thermogravimetric analysis of the

polysaccharide was done using TA Q5000IR TGA apparatus using 15 mg 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. As for pyrolysis GC/MS analysis, samples (0.1 to 1 mg) were pyrolyzed in a quartz holder with a heated-filament pyrolyzer (Pyroprobe 1000) at 600 ^{o}C for 10 s. The pyrolyzer was connected to a HP 6890 GC equipped with a HP 5973 quadrupole MSD analyzer, which in turn was coupled to an ion trap detector (Magnum; Finnigan). The GC column was a DB-5 fused silica capillary column (30×0.25 mm i.d.× 0.25 µm d.f., J and W Scientific) for the separation. The GC oven was programmed to operate from 50 to 280 °C at 5 °C /min. The injector was at 250 ^{o}C in the split mode (1/50 split ratio). One microliter of product sample was injected onto the column and the components eluting from the column were analyzed using MS. Mass spectra were recorded under electron impact at 70 eV from 30 to 550 m/Z (1 scan per s). Compound-identification was done by comparing the standard mass spectra with in NIST02 library and other references [10].

Antioxidant Activity Assays

For the evaluation of antioxidant activity of polysaccharide extracted from tobacco leaves, DPPH Radical scavenging activity and OH radical scavenging activity were determined according to the methods of Eloff *et al*. [11] and Wang *et al*. [12], 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).

Statistical Analysis

Data were expressed as mean ± S.D. (n = 3). The statistical significance was determined by Student's t-test. Experimental results from the experimental design were statistically subjected to analysis of variance (ANOVA) 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

Optimization of Extraction Conditions by Orthogonal Matrix Method

Based on the preliminary single-factor experiment results, the orthogonal matrix $L_9(3^4)$ method was used to optimise the process of ultrasound-assisted extraction and investigate the relationships between variables of extraction factors. The experimental conditions for each project were listed in Table-2, and experimental results were also included in the last column of this table.

According to the orthogonal method [13], effect of those extraction factors on extraction yield was calculated and the results were shown in Table-2. According to the magnitude order of R (Max Dif), the order of effect of all factors on extraction yield could be determined. The order of effects of factors on extraction yield was in the order of ultrasonic power > extraction temperature > extraction time > ratio of water to raw material. This result pointed out that the effect of ultrasonic treatment was more important than other factors. To obtain the optimization levels of each factor, the optimum extraction conditions are $A_2B_3C_2D_2$, that is, ultrasonic power of 500 W, extraction temperature of 50 ^{o}C, extraction time of 8 min and ratio of water to raw material of 25:1 (mL/g). Through confirmatory test, consequently 3.12% extraction yield was obtained under the above optimum conditions. This implied that the selected conditions were really the most suitable.

Table-2: Application and analysis of $L_9(3^4)$ orthogonal projects to pectic polysaccharide extraction from the oriental tobacco leaves.[a]

Run	A: Power (W)	B: Time(min)	C: Ratio of Solvent to Solid	D: Temperature (^{o}C)	Extraction yield (%)
1	1(400)	1(4)	1(20)	1(40)	2.47±0.035
2	1(400)	2(6)	2(25)	2(50)	2.87±0.032
3	1(400)	3(8)	3(30)	3(60)	2.78±0.018
4	2(500)	1(4)	2(25)	3(60)	2.99±0.013
5	2(500)	2(6)	3(30)	1(40)	2.75±0.029
6	2(500)	3(8)	1(20)	2(50)	3.08±0.011
7	3(600)	1(4)	3(30)	2(50)	2.64±0.021
8	3(600)	2(6)	1(20)	3(60)	2.74±0.016
9	3(600)	3(8)	2(25)	1(40)	2.72±0.027
k_1	2.71 [a]±0.21	2.70±0.27	2.76±0.31	2.65±0.15	
k_2	2.94±0.17	2.79±0.07	2.86±0.14	2.86±0.22	
k_3	2.70±0.05	2.86±0.19	2.72±0.07	2.84±0.13	
Optimal level	2	3	2	2	
R	0.23[b]	0.16	0.14	0.22	

[a]The arrangements of column A, B, C and D were decided by orthogonal design for 4 (factor) ×9 (run number); every row of run number represents one experimental replicate, every run was replicated twice.

[a] k_i^A= (Σ extraction yield at A_i)/3

[b] R_i^A= max $\{k_i^A\}$ − min$\{k_i^A\}$.

Isolation, Purification and Analysis of Carbohydrates

The polysaccharide was obtained from the oriental tobacco leaves by the method of ethanol precipitation. In gel filtration chromatography of the culture filtrate on Sepharose CL-6B, two fractions (designated as Fr-I and Fr-II) of polysaccharide were coeluted as shown in Fig. 1. This is in agreement with the results reported by Zhu and Tao (1993) for leaves of *Nicotiana tabacum* L [14]. They examined that two fractions were obtained by SephadexG-75 chromatography. The detailed monosaccharide compositions of carbohydrate in the two polysaccharide fractions by the trifluoroacetic acid hydrolysis and GC-MS analysis method are illustrated in Table-3. The result indicated that Fr-I was mainly composed of mannose (21.89 %), galactose (16.07 %) and ribose (14.28 %), while Fr-II was mainly composed of galactose (21.19 %), glucose (18.43 %), allose (14.18 %) and mannose (14.07 %). However, one polysaccharide isolated from burley tobacco was reported to be composed of galactose, arabinose, glucuronic acid and rhamnose [15].

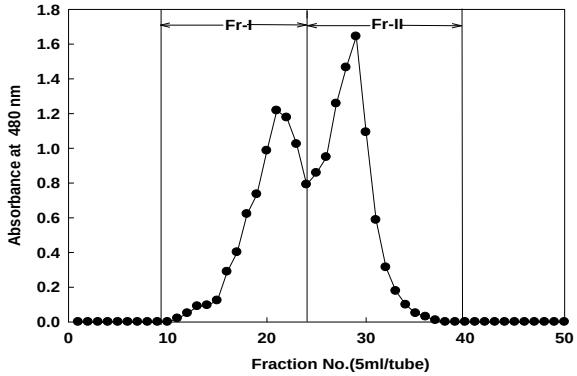

Fig. 1: Elution profiles of the pectic polysaccharide in Sepharose CL-6B chromatography. Elutes were analyzed by measuring the absorbance at 490 nm for carbohydrate.

Table-3: Carbohydrate composition in the purified pectic polysaccharide fractions (Fr-I and Fr-II) extracted from the oriental tobacco leaves.

Carbohydrate composition (%)	Fr-I	Fr-II
D-deoxyribose	2.46	0.69
D-arabinose	9.62	6.04
L-rhamnose	10.55	8.26
D-ribose	14.28	6.4
D-(-)-lyxose	1.45	0
D-xylose	5.93	7.68
D-(+)-tarot pyranose	1.19	1.45
D-(+)-tarot furanosyl	4.05	1.26
D-(+)-galactose	16.07	21.19
D-glucose	4.47	18.43
D-(+)-mannose	21.89	14.07
D-(+)-galacturonic acid	2.18	0
D-allose	3.74	14.18
D-Glucuronic acid	2.11	0

FT-IR Spectroscopy

FT-IR is an effective analytical instrument for characterizing covalent bonding information and detecting functional groups. Typical IR spectra for the two polysaccharide fractions were presented in Fig. 2. All samples exhibited a broad stretching intense characteristic peak at approximately the region of 3280 cm^{-1} for the carbohydrate ring, and a weak C–H band at around 2928 cm^{-1}. A characteristic absorption band appeared at 1589.0-1601.4 cm^{-1} was assigned to the stretching vibration of the carboxyl group (C=O) of the polysaccharide. The stretching vibration peaks of around 1031.8-1040.5 cm^{-1} suggested the presences of C-O-H link bond position [16]. The characteristic absorption at 890.3 cm^{-1} was found in the IR spectrum of Fr-I, indicated α-configuration of the sugar units [17].

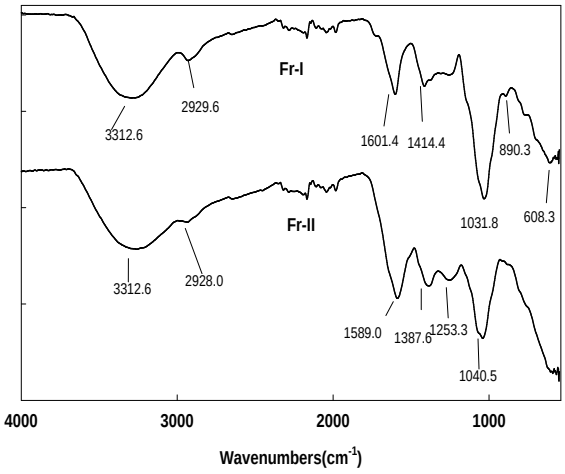

Fig. 2: The FT-IR spectra of the pectic polysaccharide fractions (Fr-I and Fr-II) from oriental tobacco leaves.

Thermal Analysis of Polysaccharide

Thermogravimetric analysis involves measuring a sample's change in mass with variation of temperature and is a very useful technique for analyzing samples that either gain or lose mass during heating. 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 215 ^{o}C, and 162 ^{o}C, respectively. This fact suggests that the material should not be submitted to the temperature of Td in order not to compromise the physical integrity of the material

evaluated. Furthermore, the weight of each fraction was dramatically lost around 240 ^{o}C and continued gradually to decrease and the final residue was 1.68 % for Fr-I and 32.93 % for Fr-II. Through this analysis, it appears that the two fractions from oriental tobacco leaves possess high thermal stability. Furthermore, the Fr-I and Fr-II have different behaviour to degradation, probably due to their different chemical compositions.

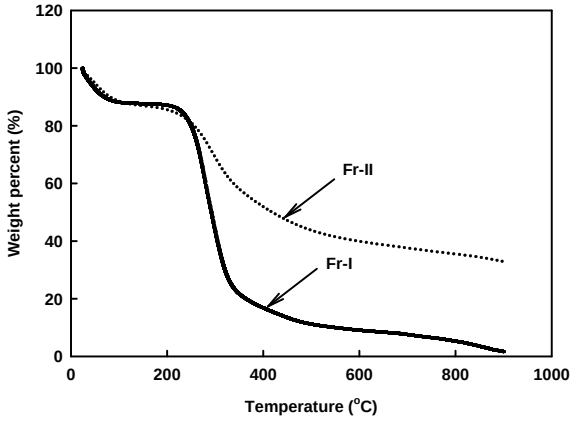

Fig. 3: TGA thermogram of pectic polysaccharide fractions (Fr-I and Fr-II) from oriental tobacco leaves.

The pyrolytic behavior is of great importance for the better understanding of the pyrolysis and thermochemical stability of polysaccharide. Py-GC/Ms is widely used techniques for characterizing the structural features on pyrolysates of polymeric materials, such as lignin and polysaccharide in plant. Py–GC/MS is based on depolymerisation of the macromolecules by heat followed by identification of the fragments by mass spectrometry. Thus, the pyrolysis products are mainly depended on the compositions of substance [18]. Table-4 shows the thermal degradation products resulting from filament Py-GC-MS of Fr-I and Fr-II, respectively. As showed in Table-4, the main pyrolysis products of both Fr-I and Fr-II included ketones, acids, esters, phenolic, aomatic, aldehydes and others. However, the pyrolysis fragments derived from the Fr-II were significantly more abundant than those from Fr-I, particularly the amounts of heterocycle and aromatic compounds, whereas the highest percentage of pyrolysis fragments (i.e. 2,3-Dimethyl-oxirane and 1-Hydroxy-2-propanone) was detected in Fr-I. The results indicate that there is a considerable variation in chemical composition between Fr-I and Fr-II.

Table-4: Pyrolysis products of the purified pectic polysaccharide fractions (Fr-I and Fr-II) extracted from the oriental tobacco leaves.

No.	Rt (min)	Name	MQ%[a]	Fr-I (%)	Fr-II (%)
1	1.89	2,3-Dimethyl-oxirane	97	9.56	-[b]
2	1.98	1,3-Cyclopentadiene	93	-	2.76
3	2.08	1-Propanol	64	2.96	-
4	2.14	2,3-Butanedione	88	5.2	-
5	2.31	Acetic acid	91	7.83	7.55
6	2.58	1-Hydroxy-2-propanone	92	9.32	3.93
7	2.81	Propanoic acid	88	-	0.7
8	2.95	3-Methyl-3-buten-2-ol	82	-	1.53
9	3.40	1-Methyl-1H-pyrrole	90	0.6	-
10	3.41	Pyridine	90	-	1.37
11	3.48	6-Methylpyridazin-3(2H)-one	70	0.76	-
12	3.58	Pyrrole	82	0.95	1.38
13	3.73	Acethydrazide	78	2.47	
14	3.77	Toluene	94	-	2.27
15	3.92	Butanedial	84	0.67	-
16	4.04	Methyl pyruvate	92	1.65	0.45
17	4.21	1-Methyl-2-pyrrolidineethanamine	80	0.91	0.5
18	4.50	3-Furaldehyde	90	0.39	0.28
19	4.59	2-Methyl-pyridine	78	-	0.35
20	4.66	4-Methyl-pyridine	86	-	0.32
21	4.71	8-Nonynoic acid, methyl ester	89	0.46	-
22	4.90	Furfural	81	2.57	1.22
23	5.06	3-Methyl-1H-pyrrole	86	-	1.29
24	5.1	3-Ethyl-1H-pyrrole	78	0.62	-
25	5.4	2-Furanmethanol	80	0.74	0.46
26	5.6	3-Methyl-pyridine	91	-	1
27	5.64	1,2-Ethanediol, diacetate	87	-	1.34
28	5.67	3,4-Epoxy-2-butanone	82	2.33	-
29	6.05	2-Cyclopentene-1,4-dione	80	0.45	-
30	6.22	1,3,5,7-Cyclooctatetraene	96		0.61
31	6.23	1,2-Ethanediol, monoacetate	82	0.52	-
32	6.53	3,5-Dimethyl-1-Hexyn-3-ol	73	0.12	-
33	6.6	2-Methyl-2-cyclopenten-1-one	90	0.42	0.83
34	6.72	1-(2-Furanyl)-ethanone	80	0.27	-
35	6.73	Butyrolactone	49	-	0.33

Table-4 continue

36	**6.78**	**1,4,5-Trimethyl-imidazole**	**74**	**-**	**0.5**
37	**6.8**	**1,3,5-Trimethyl-1H-pyrazole**	**82**	**0.94**	**-**
38	**7.07**	**1,2-Cyclopentanedione**	**86**	**1.79**	**0.32**
39	**7.11**	**1-Ethyl-1H-pyrrole**	**68**	**-**	**0.59**
40	**7.23**	**2-Cyclohexen-1-one**	**79**	**-**	**0.61**
41	**7.42**	**2,5-Dimethyl-1H-pyrrole**	**74**	**-**	**0.33**
42	**7.45**	**5-Methyl-2(5H)-furanone**	**82**	**0.19**	**-**
43	**8.01**	**(R)-3-Methyl-cyclohexanone**	**83**	**0.19**	**0.44**
44	**8.1**	**5-Methyl-2-furancarboxaldehyde**	**97**	**1.52**	**0.6**
45	**8.15**	**3-Methyl-2-cyclopenten-1-one**	**76**	**-**	**1.07**
46	**8.25**	**4-Ethyl-4-methyl-1-hexene**	**89**	**0.3**	**-**
47	**8.53**	**4-Methyl-2-oxo-(1H)-pyrimidine**	**46**	**-**	**0.31**
48	**8.54**	**4-Methyl-5H-furan-2-one**	**85**	**0.25**	**-**
49	**8.67**	**Phenol**	**93**	**-**	**4.91**
50	**8.98**	**Thiophene**	**78**	**0.63**	**0.46**
51	**9.08**	**3-Methyl-2,4-imidazolidinedione**	**50**	**0.39**	**0.89**
52	**9.21**	**(R)-3-Methyl-cyclohexanone**	**82**	**-**	**0.19**
53	**9.42**	**2-Ethyl-4-methyl-1H-pyrrole**	**86**	**-**	**0.21**
54	**9.53**	**4,6-Dihydroxypyrimidine**	**80**	**0.42**	**-**
55	**9.67**	**2,3,5-Trimethyl-1H-pyrrole**	**78**	**-**	**0.5**
56	**9.97**	**3-Methyl-1,2-cyclopentanedione**	**95**	**1.22**	**1.03**
57	**10.29**	**2-Methoxy-5-methyl-thiophene**	**78**	**-**	**0.61**
58	**10.33**	**2-Methyl-5-(methylthio)-furan**	**87**	**1.58**	**-**
59	**10.4**	**4-Ethyl-2-methyl-pyrrole**	**81**	**-**	**0.42**
60	**10.47**	**Guanazine**	**76**	**0.75**	**-**
61	**10.56**	**2,5-Piperazinedione**	**73**	**0.5**	**-**
62	**10.77**	**2-Methyl-phenol**	**91**	**0.52**	**0.51**
63	**10.97**	**1-(1H-Pyrrol-2-yl)-ethanone**	**89**	**-**	**0.14**
64	**10.98**	**4-Pyridazinamine**	**72**	**0.25**	**-**
65	**11.37**	**4-Methyl-phenol**	**95**	**0.44**	**1.77**
66	**11.75**	**2-Methoxy-phenol**	**86**	**-**	**0.4**
67	**11.89**	**N-Methyl-1,3-propanediamine**	**80**	**0.51**	**-**
68	**12.14**	**1,2-Pentadiene**	**73**	**0.23**	**0.29**
69	**12.28**	**1-(4-Pyridinyl)-ethanone**	**85**	**-**	**0.38**
70	**12.45**	**Maltol**	**91**	**1.17**	**0.72**
71	**12.61**	**1H-Imidazole-4-carboxylic acid, methyl ester**	**64**	**-**	**0.14**
72	**12.64**	**3-Ethyl-2-hydroxy-2-cyclopenten-1-one**	**92**	**0.56**	**-**
73	**12.74**	**2-Methyl-cyclobutanone**	**85**	**0.16**	**-**
74	**12.89**	**5-Hexyldihydro-2(3H)-furanone**	**92**	**0.06**	**0.19**
75	**13.19**	**Benzyl nitrile**	**89**	**-**	**0.24**
76	**13.48**	**2,4-Dimethyl-phenol**	**94**	**-**	**0.23**
77	**13.58**	**Dihydro-5-methyl-3(2H)-furanone**	**81**	**0.17**	**0.12**
78	**13.94**	**2,3-Dihydroxybenzaldehyde**	**93**	**0.15**	**-**
79	**14.01**	**4-Ethyl-phenol**	**90**	**-**	**0.43**
80	**14.08**	**2-Ethyl-phenol**	**80**	**-**	**0.12**
81	**14.2**	**N-(3-Methyl butyl)acetamide**	**74**	**0.47**	**-**
82	**14.28**	**4-Pyridinemethanol**	**78**	**-**	**0.41**
83	**14.33**	**Hydrazine, 1-methyl-1-phenyl**	**62**	**0.45**	**0.44**
84	**14.55**	**Unknown**		**2.21**	**-**
85	**14.6**	**4-Butoxy-1-butanol**	**75**	**-**	**0.28**
86	**15.06**	**1,2-Benzenediol**	**93**	**0.48**	**4.63**
87	**15.49**	**2,3-Dihydro-benzofuran**	**80**	**-**	**0.49**
88	**15.88**	**6-Methyl-3(2H)-pyridazinone**	**78**	**-**	**0.48**
89	**16.64**	**Isoquinoline**	**79**	**-**	**0.37**
90	**17.07**	**o-Isopropyl-benzenethiol**	**90**	**0.16**	**-**
91	**17.18**	**2,3-Dihydro-1H-inden-1-one**	**96**	**0.12**	**0.28**
92	**17.23**	**Hydroquinone**	**93**	**-**	**0.57**
93	**17.57**	**Indole**	**86**	**0.14**	**0.87**
94	**17.91**	**Cinnamyl alcohol**	**94**	**0.37**	**0.3**
95	**18.11**	**2-Methoxy-4-vinylphenol**	**91**	**-**	**0.07**
96	**18.51**	**2-(Dimethylamino)-phenol**	**87**	**-**	**0.17**
97	**18.61**	**3-(Methylthio)-pyridine**	**82**	**-**	**0.17**
98	**19.07**	**Unknown**	**95**	**0.46**	**1.09**
99	**20.02**	**6-Methyl-1H-indole**	**91**	**-**	**1.54**
100	**20.92**	**1,3-Dimethyl-naphthalene**	**86**	**-**	**0.18**
101	**20.99**	**Myosmine**	**95**	**-**	**0.26**
102	**22.57**	**Unknown**	**80**	**0.48**	**1.51**
103	**22.64**	**2,3-Dimethyl-1H-indole**	**94**	**-**	**0.7**
104	**23.65**	**2,3'-Dipyridyl**	**89**	**-**	**0.18**
105	**24.38**	**Dodecanoic acid**	**92**	**-**	**0.16**
106	**24.85**	**5-Phenyl-1,4-dimethyl-imidazole**	**91**	**-**	**0.21**
107	**27.64**	**(1,1'-Biphenyl)-3,3'-diol**	**38**	**0.19**	**0.22**
108	**31.01**	**1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester**	**86**	**0.43**	**0.25**
119	**32.78**	**n-Hexadecanoic acid**	**90**	**0.13**	**0.09**
110	**32.86**	**Dibutyl phthalate**	**94**	**0.39**	**0.3**
111	**38.36**	**Tributyl acetylcitrate**	**94**	**0.42**	**-**

[a]MQ% = match quality.
[b]not detected.

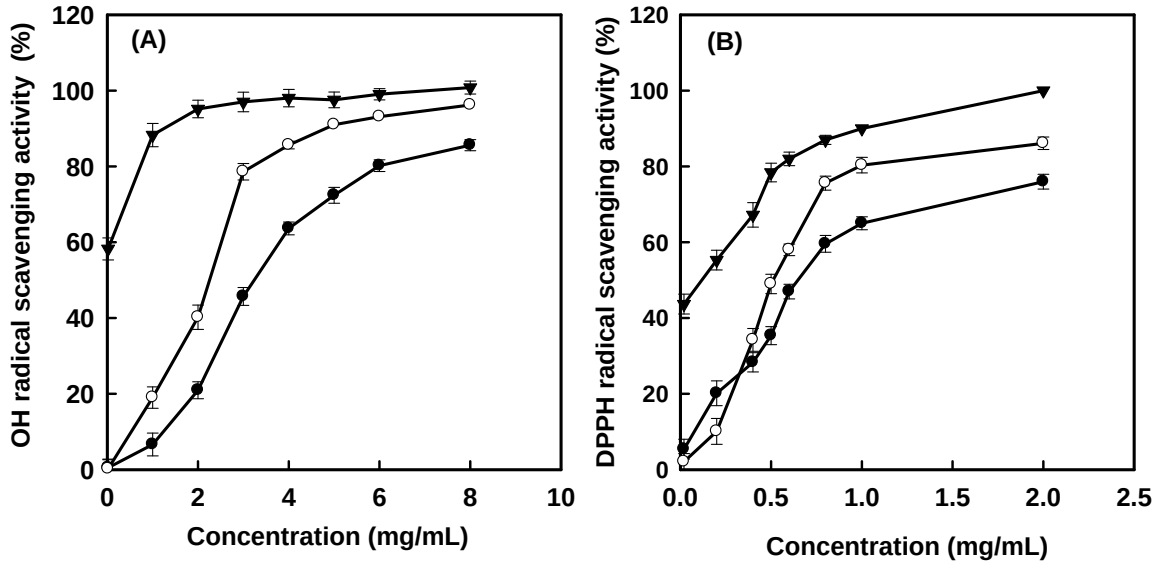

Fig. 4: Antioxidant activity of pectic polysaccharide fractions (Fr-I and Fr-II) from oriental tobacco leaves. The results represent mean ± S.D. (n = 3). OH (A) and ABTS (B) radical scavenging activity of polysaccharide. Vitamin C (▼), Fr-I (●) and Fr-II (○).

Antioxidant Properties Analysis

In this experiment, the *in vitro* antioxidant capacities of two fractions from the oriental tobacco leaves were evaluated using different biochemical methods including hydroxyl and DPPH radical scavenging assay. Hydroxyl radical can react with most biomacromolecules functioning in living cells and induce severe damage to the adjacent biomolecules [12]. The results of hydroxyl radical scavenging activities of two polysaccharide fractions are shown in Fig. 4A. The results showed that the hydroxyl radical scavenging activities enhanced corresponding to increase with the concentration. The ability of Fr-II was stronger than that of Fr-I at every concentration point and the OH radical scavenging rate at 8 mg/mL was 96.2 % (closed to scavenging activity of vitamin C). DPPH radical is a widely used method to evaluate the free radical scavenging ability of natural compounds [19]. In this experiment, scavenging rates of Fr-I and Fr-II on DPPH free radical were showed in Fig. 4B. The results showed that the EPS increased the radical scavenging activity in a concentration dependant manner. The scavenging activities of Fr-II were stronger than those of Fr-I, and finally reached to 86.1 % at the concentration of 2.0 mg/mL.

In this experiment, both polysaccharide fractions showed significant antioxidant effects. However, the Fr-II has stronger antioxidant activities than Fr-I, which probably attribute monosaccharide compositions. This is in accordance with the report by Fan *et al*. for the polysaccharide extract from *Dendrobium denneanum* [20]. They suggested that the antioxidant abilities of the polysaccharide fractions of *D. denneanum* were supposed to relate to the configuration of the sugar units and chemical compositions.

Conclusions

As a more practical method, the orthogonal matrix method was employed to study the relationships between the extraction condition and effect on extraction yield. The factors of UAE on the yield of polysaccharide from oriental tobacco leaves were studied by using orthogonal matrix method. The results showed that an optimum yield of polysaccharide could be obtained when conditions were set as follows: ultrasonic power was of 500 W, extraction temperature of 50 ^{o}C, extraction time of 8 min and ratio of water to raw material of 25:1 (mL/g). The crude polysaccharide was purified with Sepharose CL-6B column, and two components were obtained (Fr-I and Fr-II). The characterization of Fr-I and Fr-II was analysed with FT-IR spectroscopy, GC, TGA and Py-GC/MS analysis. Furthermore, both polysaccharide fractions showed strong antioxidant activity.

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