doi.org/10.36721/PJPS.2020.33.2.REG.581-588.1

Ultrasonic-assisted extraction and antioxidant activity of polysaccharides from *Eucommia ulmoides* leaf

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Abstract: *Eucommia ulmoides* is valuable medicinal plant in China. In this study, ultrasonic technology was used to extract polysaccharides and orthogonal design was applied to choose the optimal extraction conditions. The optimal extraction conditions of *E. ulmoides* polysaccharides were made up of the ratio of water to raw 30, extraction time 80 min, extraction temperature 60 °C and extraction power 200 W. Under these conditions, the extraction polysaccharides content reached 164.95 mg/g. In addition, the potential antioxidant activity of crude polysaccharides (Cp) and pure polysaccharides (Pp) was demonstrated by evaluating reducing power assay, DPPH radical-scavenging assay, OH radical-scavenging assay and ABTS radical-scavenging assay. The results showed that *E. ulmoides* polysaccharides had significantly impact on the scavenging of DPPH radicals, OH radicals and ABTS radicals, expecially in DPPH radicals with an IC_{50} values of 0.005 mg/mL and 0.011 mg/mL in Cp and Pp, respectively. However, they were less effective in reducing power assay with low IC_{50} values of 1.091 mg/mL and 1.041 mg/mL separately. These results indicated that polysaccharides from *E. ulmoides* leaf could be applied as potential antioxidant.

Keywords: *Eucommia ulmoides*, polysaccharides, ultrasonic-assisted extraction, antioxidant activity.

INTRODUCTION

Polysaccharides are natural polymers with macromolecule weight, which are widely distributed in animals, plants and microorganisms (Zhong *et al*., 2010; Yang *et al*., 2016). Polysaccharides from natural sources have significant function, such as some polysaccharides from botanicals and fungus, which may be developed as potential antioxidants (Hua *et al*., 2012; Jin *et al*., 2012). Recently, many studies have focused on the polysaccharides obtained from natural Chinese herbal medicine. Due to their significant pharmacological actions, they can be used in the fields of pharmacology and explored into drugs, such as antioxidant (Jing *et al*., 2015), antiinflammatory activities (Chen *et al*., 2015), immunological activities (Yu *et al*., 2015). With the development of cytobiology and immunology, search for polysaccharides with few side effects has become an important biomedical research content (Wang *et al*., 2014b).

Eucommia ulmoides Oliv. is valuable medicinal plant in China. It accounts for about 99 % of the world's total resources (Du, 1996). The *E. ulmoid*es leaf has typical characteristics for its glue which has great potential in the development of rubber. In addition, it contains a variety of physiological activity compounds, such as aucubin, geniposidic acid, chlorogenic acid, asperuloside and geniposide. Polysaccharides is another important compounds in *E. ulmoid*es leaf (Zhao *et al*., 2008) (fig. 1). So far, about *E. ulmoides* polysaccharides, little research has been done. Two kind of acidic polysaccharides named eucommia A and B were isolated from *E. ulmoides* bark by Japanese scholars. Meanwhile, it was proved that the polysaccharides could enhance the body's nonspecific immune function through the structure analysis and pharmacological research (tomoda *et al*., 1990). In addition, it was found that *E. ulmoides* leaf had the effect of anticomplement through the pharmacological experiments (Zhang *et al*., 2011).

The lower polysaccharides extraction yield from *E. ulmoides* leaf is one of the main problems to hinder its development. Therefore, in this study, ultrasound-assisted technology was applied to extract polysaccharides and orthogonal design was used to choose the optimal extraction conditions. In addition, the potential antioxidant activity of crude polysaccharides (Cp) and pure polysaccharides (Pp) was demonstrated by evaluating reducing power assay, DPPH radical-scavenging assay, OH radical-scavenging assay and ABTS radical-scavenging assay, which could provide references for the development of *E. ulmoides* polysaccharides.

MATERIALS AND METHODS

Materials and reagents

Leaf was collected from *E. ulmoides* research base of Chinese Academy of Forestry. Reagents of DPPH (1,1-

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Diphenyl-2-picryl-hydrazyl) and ABTS [2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulph-onicacid) acid diammonium salt] were bought from Sigma. gallic acid, ascorbic acid (Vc), salicylic acid and so on were obtained from Beijing chemical reagent company. The other reagents used in this study were of analytical grade.

Extraction of polysaccharides

E. ulmoides leaf was dried to constant weight in thermostatic blower dryer at 50 °C, then comminuted and filtered by 60 mesh sieve. Ultrasound-assisted extraction technology was used to prepare polysaccharides, and the orthogonal test was applied to optimize the four selected factors (ratio of water to raw (v/w), extraction time (min), extraction temperature (°C), extraction power (W)) for enhancing the polysaccharides extraction yield of *E. ulmoides* leaf (%). The four independent factors were researched at three different levels (1, 2, 3). After centrifugation, the supernatant liquid was crude polysaccharides (Cp). As the polysaccharides content for evaluation index, the best extraction condition was found. The experimental design used in this study was shown in table 1.

The polysaccharides content was determined using phenol-sulfuric acid method with little modification (Fukuda *et al*., 1975). In a tube, 2 mL extraction solution was added first, following by 1mL of 6 % phenol, and then 5 mL of concentrated H_2SO_4 was added quickly. The solution was shaked slowly for 10min. After that, the absorbance was measured using UV-Vis spectrophotometer at 490 nm. Water was used as a positive control. Standard calibration curve was drawn by glucose solutions.

Purification of polysaccharides

Hierarchical alcohol sink

The Cp was precipitated with the 30%, 40%, 50%, 60%, 70%, 80% absolute ethanol for hierarchical sink at 4°C for 24h respectively, and then centrifuged to get the polysaccharides precipitate.

Sevag assay and decolorization

The polysaccharides precipitate was dissolved. Sevag method was used to take off its protein according the Li *et al*. (2015). In decolorization, the hydrogen peroxide and active carbon methods were adopted to compare, and the final concentration of each was 5 %, 10 %, 20 %, 30 %, 40 %, respectively.

Anion-exchange chromatography

The Cp was loaded into a DEAE-52 anion-exchange chromatography column first, and then cleared separately by ultrapure water and 0.1 mol/L of NaCl solution with a slow rate10 mL/h. The eluent was collected and analyzed using the same method as 2.2. The eluting graph was drawn, of which X axis was tube number and Y axis was absorbance at 490 nm. According to the graph, the major peak was collected, dialyzed, lyophilized, and named as pure polysaccharides (Pp).

Antioxidant activity

Reducing power assay

The reducing power of Cp and Pp was detected using the method described by Chen *et al*. (2015) with slightly change. 1mL extract liquid of different concentrations were mixed with 2.5mL of 1% potassium ferricyanide and 2.5 mL of 0.2 M phosphate buffer (pH 6.6), and then incubated 20 min at 50°C. 5 mL of 10% trichloroacetic acid was added to the mixture and centrifuged 10min with 5000 rpm. At last, 2.5 mL of the supernatant solution was taken out and mixed with 0.5 mL of 0.1 % ferricchloride and 2.5 mL of distilled water to react 30 min. The reaction liquid was measured at 700 nm using UV-Vis spectrophotometer, with Vc as a positive control (0.04 mg/mL ~ 0.18 mg/mL).

DPPH radical-scavenging assay

DPPH had been widely used to evaluate reducing substances as a free radical (Wu *et al*., 2014). In this paper, DPPH radical-scavenging ability of Cp and Pp was determined by the method (Balavigneswaran *et al*., 2013) with slight modification. 2.0 mL samples at different concentrations were blended with 2.0 mL of methanol solution including DPPH radicals with a concentration of 0.2 mM. The blender was mixed fully and reacted for 30 min. The finally extration liquid was detected by UV-Vis spectrophotometer at 517 nm, using Vc as a positive control. The DPPH scavenging activity was calculated according to the following equation:

$$\text{Scavenging rate}\,(\%) = \frac{A_0 - (A_1 - A_2)}{A_0} \times 100$$

Where A_0 as the absorbance of a control, A_1 as the absorbance of the samples and A_2 as the absorbance of samples background.

OH radical-scavenging assay

The OH radical-scavenging activity was detected according to the method reported by Li *et al*. (2015) with minor modifications. 3.0mL reaction mixture included 1mL of 6 mM sodium salicylate, 0.5 mL of 8.8 mM hydrogen peroxide, 1.0 mL of 6 mM FeSO4 and 0.5 mL of different concentration samples. The mixture reacted at 37°C for 1h, and then the absorbance was measured by UV-Vis spectrophotometer at 510nm, with Vc as a positive control. The OH radical-scavenging rate was expressed as following equation:

$$\text{Scavenging rate}\,(\%) = \frac{B_0 - (B_1 - B_2)}{B_0} \times 100$$

Where B_0 as the absorbance of a control, B_1 as the absorbance of the samples, and B_2 as the absorbance of samples background.

ABTS radical-scavenging assay

The ABTS radical-scavenging ability was measured according to the method of Katalinic *et al*. (2006) with minor change. The ABTS radical cation ($ABTS^+$) was generated through mixing 7 mM of ABTS solution with 2.45 mM potassium persulfate aqueous solution for reacting 16 h in dark. Then 0.1 mL of different concentrations samples were mixed with 3.0 mL of the $ABTS^+$ solution. The mixture reacted 20 min and then the absorbance was detected by UV-Vis spectrophotometer at 734 nm, using Vc as a positive control. The ABTS radical-scavenging rate was calculated using following equation:

$$\text{Scavenging rate}\,(\%) = \frac{C_0 - (C_1 - C_2)}{C_0} \times 100$$

Where C_0 as the absorbance of a control, C_1 as the absorbance of the samples, and C_2 as the absorbance of samples background.

FT-IR analyses

The Pp spectrum was measured by Fourier Transform Infrared Spectrophotometer (FT-IR). 1~ 2 mg Pp was ground with potassium bromide (KBr) powder and pressed into pellets, and then detected with the frequency range of 4000-400 cm^{-1}.

STATISTICAL ANALYSIS

All data were showed using means ± standard deviations (SD) of three replicated determinations. Analysis of variance (ANOVA) was performed using the software SPSS 18.0, and P-Values of less than 0.05 were regarded as statistically significant.

RESULTS

Optimization of the extraction condition

Different experimental factors combinations made the content of Cp different. Through range analysis (table 1), the optimum extraction conditions were made up of the ratio of water to raw 30, extraction time 80 min, extraction temperature 6 0°C and extraction power 200 W. Under the optimum conditions, the extraction polysaccharides content had reached 164.95 mg/g.

The F-test and P-values were used to evaluate statistical significance of each coefficient. The smaller the P values was, the more significant of the corresponding coefficient was. The ANOVA results of orthogonal test were shown in table 2. The coefficient of ratio of water to raw and extraction time were highly significant with small P values ($P<0.01$), and the coefficient of extraction temperature was significant with P values less than 0.05. However, the coefficient of extraction power was found to be non-significant for the P values more than 0.05.

Process of polysaccharides purified

In the process of polysaccharides purified, hierarchical alcohol sink method was used to improve the polysaccharides yield. In this experiment, the final concentration of absolute ethanol was 30 %, 40 %, 50 %, 60%, 70%, 80% in turn. As extraction rate for evaluation index, the yield of polysaccharides increased with the concentration of absolute ethanol improved (Table 3). When the concentration of absolute ethanol was added from 30% to 70%, the increase polysaccharides yield was between 12.3% and 36.9%. Whereas, when the concentration of absolute ethanol reached 80%, the increase yield was just 0.57%. So the best ethanol precipitation method was that its concentration eventually reached 70%, which made the polysaccharides yield to from 4.82% to 10.49%. In addition, two methods were adopted to compare the ability of decolorization. The effect of hydrogen peroxide method was better. When its final concentration reached 20%, the solution color of polysaccharides was very light. However, using active carbon method, the color of polysaccharides was still deep after its concentration for 40%. Moreover, anion-exchange chromatography was the important way to purify Cp. After this procedure, Pp could be obtained.

Fig. 1: The morphology of *E. ulmoides* leaf

Antioxidant activity of Cp and Pp

Reducing power activity

Multiple mechanisms were used to explain the antioxidant activities, such as preventing continued hydrogen abstraction and chain initiation, decomposing peroxide and chelating transition metal ions. Antioxidant polysaccharides may be defined as electron donors, which could react with free radicals (Jin *et al*., 2012; Sun *et al*., 2010). A compound with stong reducing power could be applied as a potential antioxidant. Through donating electrons, antioxidant substances were capable of blocking radical chain reaction by converting reactive oxygen species to more stable products (Arabshahi-Delouee and Urooj, 2007). The reducing power of Cp, Pp and Vc was compared in fig. 2A. Higher absorbance value indicated stronger reducing power. When the concentration was between 0.04 mg/mL and 0.18 mg/mL, the maximum reducing power of Cp and Pp was obtained at 0.16 mg/mL (absorbance = 0.255) and 0.18 mg/mL (absorbance = 0.165), and the maximum reducing power of Vc was got at 0.16 mg/mL (absorbance = 0.755). The result showed that the absorbance of Cp and Pp improved with the increasing polysaccharides concentration, and Cp was higher than Pp. The IC_{50} of Cp and Pp was separately 1.091 mg/mL and 1.041 mg/mL, which was significantly lower than that of Vc at 0.007 mg/mL. Therefore, the reducing power of Cp and Pp was in a lower level.

DPPH radical-scavenging activity

The DPPH radical-scavenging model was widely used in estimating the free radical scavenging ability of various antioxidants, which was mainly because of its hydrogen-donating ability (Yuan *et al*., 2008; Kedare and Singh, 2011). As Vc for positive control, the DPPH radical-scavenging activity of Cp and Pp was showed in fig. 2B. DPPH radical-scavenging activity of Cp and Pp was detected with different extraction concentrations. Under the concentration from 0.04 mg/mL to 0.18 mg/mL, the maximum scavenging activity of Cp and Pp was 83.3% and 80.7% at 0.18 mg/mL in turn and Vc was 95.6% at 0.18 mg/mL. The DPPH radical-scavenging activity of Cp and Pp was related well with its concentrations, and Cp was higher than Pp, but they were all lower than that of Vc on the identical concentration. The result indicated that Cp and Pp had an obviously effect on the scavenging of free DPPH radicals with an IC_{50} values of 0.005 mg/mL and 0.011mg/mL, respectively.

OH radical-scavenging activity

The OH radical, as a highly potent oxidant, belonged to reactive oxygen radicals, which could easily induce cell oxidative damage, such as destroying the most biomolecule structures including DNA, proteins and lipids, and inducing tissue damage to make cell death. Therefore, OH radical-scavenging was especially important in protecting living systems (Zhong *et al*., 2010). Fig. 2C showed the OH radical-scavenging ability of the Cp, Pp and Vc. With concentration increased, the scavenging ability of them improved. Cp and Pp had stronger OH radical-scavenging activity than Vc in the same concentration, and OH radical-scavenging activity of Cp was the strongest in the three indexs. When the concentration was between 0.04 mg/mL and 0.18 mg/mL, the maximum OH radical-scavenging activity of Cp and Pp was 75.1% and 44.0% at 0.18 mg/mL respectively, which were higher than that of Vc with 18.3%. Moreover, the Cp and Pp were found to possess OH radical-scavenging activity with an IC_{50} values of 0.072 mg/mL and 0.261 mg/mL, respectively.

ABTS radical-scavenging activity

ABTS assay was generally accepted method in detecting total antioxidant power of potential antioxidant (Jin *et al*., 2012). The scavenging ability of Cp, Pp and Vc on ABTS free radicals was shown in fig. 2D. The scavenging power of them related well with the increasing concentrations from 0.04 mg/mL to 0.18mg/mL. The ABTS radical-scavenging activity of Cp and Pp was lower than Vc when the concentration was above 0.14mg/mL. The maximum ABTS radical-scavenging of Cp and Pp was 80.1% and 52.7% at 0.18 mg/mL respectively, which were lower than Vc with 97.7%. It was showed that Cp and Pp had strong scavenging power in ABTS radicals with more than 50 % when the concentration reached 0.18mg/mL. The IC_{50} values of Cp and Pp was 0.094 mg/mL and 0.177 mg/mL respectively, which was slightly lower than that of Vc at 0.090 mg/mL. The results indicated that ABTS radical-scavenging could be applied as an index in reflecting the antioxidant activity of experimental materials (Mateos-Aparicio *et al*., 2010).

FT-IR spectrum of Pp

As shown in fig. 3, the FT-IR spectra of Pp contained typical absorption peaks of polysaccharides. The broad and strong band around 3417 cm^{-1} was due to the O-H stretching vibration, and the bands around 2930 cm^{-1} and 1406 cm^{-1} were attributed to C-H stretching vibration and bending vibration respectively. The peak at 1591 cm^{-1} was related to the stretching vibrations of C=O. In the FT-IR spectrum of Pp, a strong band between 950 cm^{-1} and 1160 cm^{-1} was because of the stretching vibrations of pyranose ring (Zhang *et al*., 2014). The absorption at 866 cm^{-1} belonged to the α-anomeric configuration. However, there was a strong band at 2142 cm^{-1} and 2050 cm^{-1} in the FT-IR spectrum of Pp, which maybe belong to the peak of protein. Sevag assay was a common and traditional method to remove protein from crude polysaccharides (Wang *et al*., 2014a). but it maybe not be very suitable for *E. ulmoides* polysaccharides. Yang *et al*. (2016) reported that the polysaccharides loss rate was above 20 % using Sevag reagent, which could also affect the completeness of polysaccharides information, such as component and structural information. Therefore, a proper method should be chosen for removing protein from the crude polysaccharides of *E. ulmoides*.

DISCUSSION

Ultrasonic-assisted extraction technology has been extensive used in the extraction of target compounds. It allows effective mass transfer between immiscible phases by super agitation at low frequencies (Hromadkova and Ebringerová, 2003; Li *et al*., 2016). Moreover, this technique also has the advantage of reproducible, low energy inputs and short extraction time (Liao *et al*., 2015). Thus, ultrasound-assisted method was used to evaluate the multiple factors in this study. Meanwhile, the orthogonal test was applied to optimize four selected factors: Ratio of water to raw, extraction time, extraction temperature and extraction power, which all played a very important role in the polysaccharides extraction of plant materials (Jin, 2012; Zhao *et al*., 2015). Moreover, in this study, it showed that the Cp had higher scavenging activity than Cp in the four antioxidant activity indexs. The reason was that Cp had some inpurity, such as flavone, polyphenol, which also had free radical scavenging abilities.

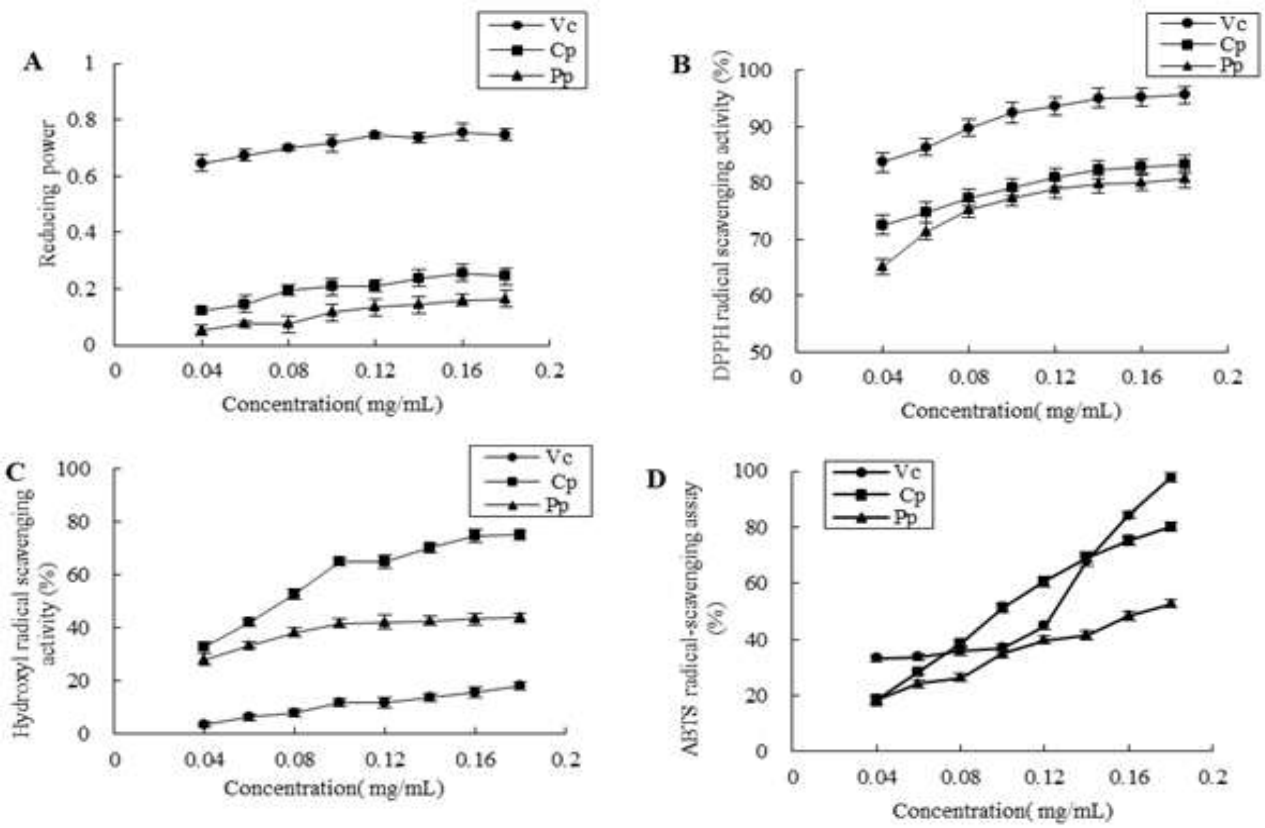

Fig. 2: Antioxidant activity of Cp and Pp from *E. ulmoides* leaf

(A: Reducing power; B: DPPH radical-scavenging ability; C: OH radical-scavenging ability; D: ABTS radical-scavenging ability)

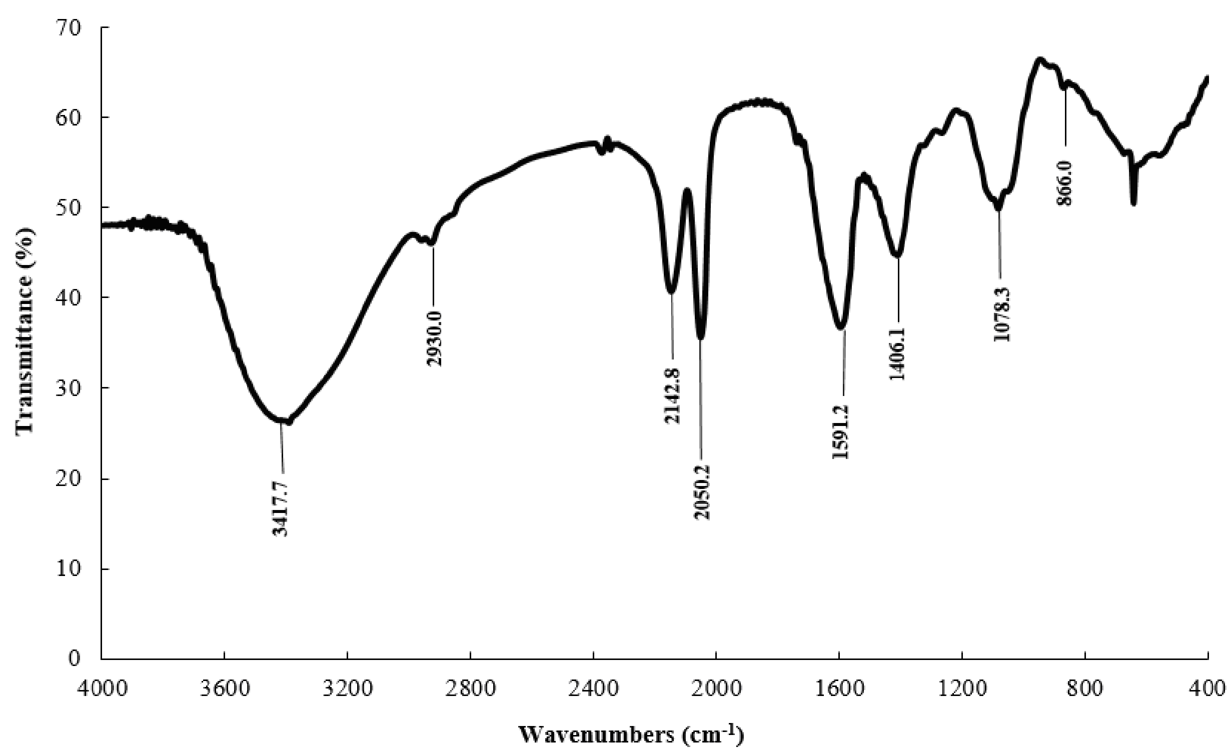

Fig. 3: FT-IR spectrum of Pp from *E. ulmoides* leaf

Table 1: L_9 (3^4) orthogonal test and range analysis

Groups	Ratio of water to raw (v/w)	Extraction time (min)	Extraction temperature (°C)	Extraction power(W)	Average Cp content (mg/g)
1	1(20)	1(60)	1(40)	1(200)	102.82
2	1	2(80)	2(50)	2(300)	108.70
3	1	3(100)	3(60)	3(400)	123.89
4	2(30)	1	2	3	111.55
5	2	2	3	1	164.95
6	2	3	1	2	134.29
7	3(40)	1	3	2	90.53
8	3	2	1	3	100.78
9	3	3	2	1	101.68
k1	111.80	101.63	112.63	123.15	
k2	136.93	124.81	107.31	111.17	
k3	97.66	119.95	126.46	112.07	
R	39.27	23.18	19.15	11.98	

Table 2: ANOVA for the effects of ratio of water to raw, extraction time, extraction temperature and extraction power on Cp content

Source	Sum of squares	df	Mean square	F-test	P-values	Significant Model
Model	1.24×10^4	8	1.55×10^3	9.39	0.000	**
Ratio of water to raw	7.12×10^3	2	3.56×10^3	21.61	0.000	**
Extraction time	2.69×10^3	2	1.34×10^3	8.16	0.003	**
Extraction temperature	1.76×10^3	2	0.88×10^3	5.34	0.015	*
Extraction power	0.80×10^3	2	0.40×10^3	2.43	0.116	
Error	2.96×10^3	18	0.16×10^3			
Total	3.75×10^5	27				

*Significant ($p<0.05$); **significant ($p<0.01$)

Table 3: Polysaccharides yield based on hierarchical alcohol sink

Ethanol content (%)	Polysaccharides weight (g)	Polysaccharides yield (%)	Polysaccharides cumulative yield (%)
30	0.2412	4.82	4.82
40	0.0889	1.78	6.60
50	0.0760	1.53	8.13
60	0.0605	1.21	9.34
70	0.0576	1.15	10.49
80	0.0032	0.06	10.55

CONCLUSION

In this study, the optimum extraction conditions of *E. ulmoides* polysaccharides consisted of ratio of water to raw 30, extraction time 80 min, extraction temperature 60°C and ultrasound power 200 W. Under these conditions, the extraction polysaccharides content reached 164.95mg/g. In addition, the results also showed the Cp and Pp of *E. ulmoides* leaf had significant impact on the scavenging of DPPH radicals, OH radicals and ABTS radicals, expecially in DPPH radicals. However, they were less effective in reducing power assay. In those four antioxidant assays, the Cp displayed prominent antioxidant activities with IC_{50} values of 1.091 mg/mL, 0.005 mg/mL, 0.072 mg/mL and 0.094 mg/mL in turn, and the Pp also possessed slightly lower activities with IC_{50} values of 1.041 mg/mL, 0.011 mg/mL, 0.261 mg/mL and 0.177 mg/mL, respectively. In conclusion, this study provided an efficient method for polysaccharides extraction from *E. ulmoides* leaf, and its antioxidant capacity was verified which could be explored as a natural antioxidant.

ACKNOWLEDGMENTS

This research was financially supported by National Key R＆D Program of China (Grant No.2017YFD0600800-2); Foundation of Henan Collaborative Innovation Center of

Food Production and Safety (Grant No.FCIC201601) and Project of Key Scientific Research in Henan Colleges and University (Grant No.17B550007).

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