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Isolation of polysaccharides from *Eucommia ulmoides* leaves and their anti-complement activities

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ABSTRACT

After removal of resin and gutta percha under petroleum ether reflux, *Eucommia ulmoides* (or Duzhong) leaves were decocted with distilled water and the combined extraction solution was concentrated and fractionally precipitated by adding the one-fold, twofold and threefold volumes of alcohol to obtain three groups of crude polysaccharides, PsEUL1, PsEUL2 and PsEUL3. Bio-active assay showed PsEUL1 and PsEUL3 possessed high anti-complement activities. Of them, the group of polysaccharides, PsEUL1, with the highest anti-comlement activity, was decolourized on a macro-porous resin (S-8) column and deproteinized using Sevag reagent. After dialysis, the refined PsEUL1 was applied to a diethylaminoethyl (DEAE)-cellulose anion exchange column and three polysaccharides, $PsEUL1_1$, $PsEUL1_2$ and $PsEUL1_3$, had been isolated. The further assays of anti-complement activities indicted these isolated polysaccharides have different extent of inhibitory activities, of which $PsEUL1_3$ was the one with the highest activity and showed dose-effect relationships. The half inhibitory concentration, IC_{50}, of $PsEUL1_3$ was 210 μg/mL. The reaction with sulfuric acid and carbazole substantiated it was an acidic polysaccharide, containing 21.2% of uronic acid. The gas chromatography revealed, besides uronic acid, PsEUL13 contained L-rhamnose, D-fucose, D-arabinose, D-xylose, D-glucose and D-galactose, and their percentage composition were 11.8%, 1.6%, 37.7%, 4.2%, 10.7% and 12.8%, respectively.

KEYWORDS

Eucommia ulmoides leaf;
Acidic polysaccharide;
Isolation;
Anti-complement activities.

INTRODUCTION

After the 1960s, it was gradually awarded that polysaccharides derived from vegetable resource bear specific biological activities and found a lot of them and their derivatives are of medicinal importance in anticoagulant, antithrombotic, regulating lipids, improvement of immune function, anti-tumour, anti-radiation, and especially in immunomodulation. In regard to the anti-complement activities, many polysaccharides form

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higher plants play a large role[1], showing a balance inhibition on excessively activated complement system[2]. Within the system, proteinase molecule activated at one step can generate multiple copies of an activated enzyme later in the cascade mechanism, which in turn cleaves nonenzymatic components[3]. The nonenzymatic components, such as C3, C4, and C5, form larger fragments, i.e., C3b, C4b, and C5b, which are involved in biologic effector functions in opsonization, phagocytosis, and immunomodulation. While anaphylatoxins, the smaller molecules, C3a, C4a, and C5a, will induce the release of mediators from the mast cells and lymphocytes, which causes a variety of inflammatory diseases and tissue damage.

Figure 1 : Folium cortex eucommiae, or *Eucommia ulmoides* (or Duzhong) leaves.

Duzhong, Chinese name of *Eucommia ulmoides* Oliv, a traditional Chinese famous and precious medicine, has been used as medicine for bone and body health care and for immunity improvement in China for thousands of years. Japanese scholars Gonda R, and Tomoda M et al[4,5] isolated from the bark of *E. ulmoides* two acidic polysaccharides (*Eucommia* A and *Eucommia* B), and indicated that the two polysaccharides could enhance the body's non-specific immune function. Later, Japanese scholars Nakano M et al[6,7] also believed that the acidic polysaccharides obtained from bark of *E. ulmoides* are anti-infective agents and cerebral metabolism enhancers, and able to inhibit virus adsorption and proliferation that are resulted from the acquired immunodeficiency and to prevent HIV infection. They additionally pointed out that the acidic polysaccharides would not cause side effects even long-term administrqation. H. Zhu et al[8,9] reported that polysaccharide from the stem bark of *E. ulmoides*, a protein-bound polysaccharide, could inhibit complement activation. *E. ulmoides* Leaves contains many of chemical components that is the same as *Eucommia* bark does[10,11]. Nowadays, researches on polysaccharide from *E. ulmoides* leaf are, however, seldom. Its current studies are limited to the determination of polysaccharide level in the leaf but the pharmacological activities. *E. ulmoides* Oliv is a deciduous tree, producing a large number of leaves yearly, whereas, its bark harvest is very very limited. Polysaccharide can never be artificially synthesized now, so the research on polysaccharide from *E. ulmoides* leave has current and practical significance. In China, especially in the Zunyi district of Guizhou Province, there is a large number of Duzhong forest, from which a great deal of fallen leaves are derived, being abundant natural resources for polysaccharides from *E. ulmoides* leaves (PsEUL).

In this study, polysaccharides is extracted from *Eucommia ulmoides* Leaves and purified in order to study their anti-complement activities by a cascade mechanism of the classical pathway (CP) and alternative pathway (AP)[12].

RESULTS AND DISCUSSION

The aqueous extract from *E. ulmoides* leaves was separated by stepwise ethanol precipitation, which would divide the extracted polysaccharides of *Eucommia ulmoides* leaves (PsEUL) into three parts, PsEUL1 (0.880g), PsEUL2 (0.280g) and PsEUL3 (0.105g) according to their molecular weight. Meanwhile protein in the aqueous extract was precipitated

with polysaccharides at the same time. The determination of total carbohydrate in PsEUL1, PsEUL2 and PsEUL3 using phenol-sulfuric acid colourimetric method showed the three groups contained a great deal of impurities and contents of carbohydrates, such as polysaccharide, starch and cellulose, were only 35.9%, 32.7% and 43.0%, respectively. Protein levels were very low, being 0.8%, 2.5% and 3.0%, respectively, according to Coomassie brilliant blue colourimetric method. The results are listed in TABLE 1.

TABLE 1 : Extraction yield, carbohydrate and protein content of three precipitates by stepwise ethanol precipitation.

Group	Mass (g)	Percentage (%)	Appearance	carbohydrate content (%)	Protein content (%)
PsEUL1	0.880	69.6	Gray powder	35.9	0.8
PsEUL2	0.280	22.1	Black grume	32.7	2.5
PsEUL3	0.105	8.3	Black grume	43.0	3.0

To remove impurities and eliminate interference of impurities on the anti-complement assay, Fractions PsEUL1, PsEUL2 and PsEUL3 were decolourized on macroporous resin column (S-8 macroporous resin, column diameter 3cm and the effective height 24cm). The decolourization rate was up to 97%. Decolourization is a very important process in the purification of polysaccharides from *E. ulmoides* leaves because leaf, unlike bark, contains a great deal of tannins, chlorophyll and coloured substances which, accompaning polysaccharides, dissolv into hot water. In the experiments of decolouration, activated carbon and hydrogen peroxide were also studied on their removal of coloured substances. The decolouration rate of activated carbon was closely related to treating temperatur, up to 90.6% at 80 °C and only ~77% at 30 °C. While the decolouration of hydrogen peroxide depended on the reaction of hydrogen peroxide oxidizing coloured materials. The oxidation was unselective and so polysaccharides were oxided at the same time, resulting in the change of chemical structure of polysaccharides and the decline of bioactivity. While the decolouration rate reached 79%, the colour of product as polysaccharides were determinated using phenol-sulfuric acid method changed from magenta to black, implying the structure of polysaccharides had been modified. Therefore macroporous resins were employed (see TABLE 2) in this study for their higher decolouration rate and stable physical and chemical properties. Then these decolourized polysaccharides got rid of dissociative protein by denaturization reaction with Sevag reagent. However, Sevag reagent could not remove conjugated protein since the decolourized and deproteinized polysaccharides showed a obvious absorption at 278 nm, which, we believed, was contributed by the protein binding to polysaccharide, or glucoprotein, which was very difficult to be removed by Sevag reagent (see Figure 2).

TABLE 2 : Retention rate of saccharide and decolourizing rates of different type of macroporous resins.

Type of macroporous resin	S-8	X-5	AB-8	D101	NKA-9
Retention rate of saccharide (%)	85.3	58.6	54.2	85.0	58.8
Decolouration rate (%)	97.4	89.8	90.8	90.0	81.3

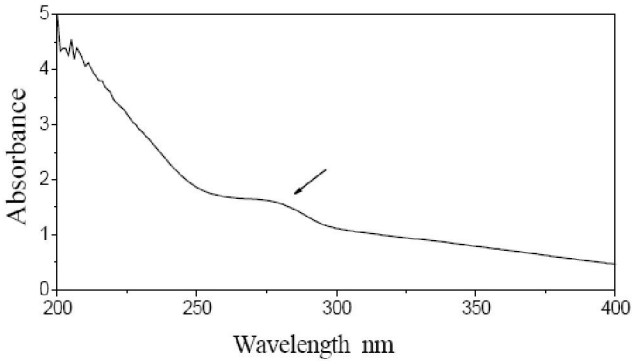

Figure 2 : UV spectrum of PsEUL1 after deproteinized and decolourized.

A classical pathway of the anti-complement assay was used to assess the bio-activities of treated polysaccharides. Of them PsEUL1 showed the highest anti-complement activity although all the inhibition rate of three polysaccharide groups were very high, see

TABLE 3 : Anti-complement activity of PsEULs

Polysaccharide group	Concentration (mg/mL)	Inhibition rate (%)
PsEUL1	3.33	99.79±0.30
	1.67	98.73±1.12
	0.33	16.80±1.27
PsEUL2	3.33	97.75±2.81
	1.67	42.47±3.70
	0.33	2.32±0.36
PsEUL3	3.33	98.87±0.76
	1.67	92.92±0.45
	0.33	24.53±2.54

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TABLE 3. The three polysaccharide groups, as the TABLE 3 suggests, exhibited a distinct ascent trend of their inhibition rate with concentration of sample solution, that is, inhibitory effects of PsEUL1, PsEUL2 and PsEUL3 on activated complement were of dose-dependent. Due to the prominence of PsEUL1 in inhibitory effect and in yield, it was selected to be subjected to a further research.

PsEUL1 that was precipitated at a condition of 50% ethanol in water contained a group of polysaccharides with a certain range of molecular weight and composed of different monosaccharide. A DEAE-52 (OH) cellulose column (2.5cm × 25cm) coupled with a automatic fraction collector was used to separated PsEUL1. NaCl solution as eluent was applied to the column and eluted the column at 0.1 mol/L, 0.2 mol/L, 0.3 mol/L and 0.4 mol/L of NaCl, respectively. The fractions obtained at different concentration of NaCl were determined using phenol-sulfuric acid method and their curves of elution were sketched in Figures 3, 4 and 5 according as their relative optical density at 490 nm. Whereas no saccharide was detected in the fractions eluted using 0.4 mol/L of NaCl. The same was true for the fractions collected under 1.0 mol/L of NaCl. So we believed the PsEUL1 comprised of only three polysaccharides, $PsEUL1_1$, $PsEUL1_2$ and $PsEUL1_3$. $PsEUL1_1$ was

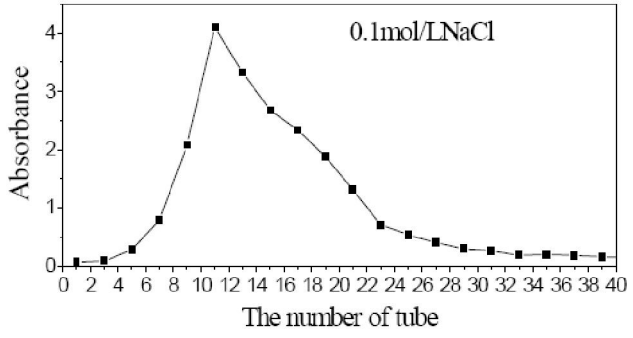

Figure 3 : Fractions eluted by 0.1 mol/L of NaCl.

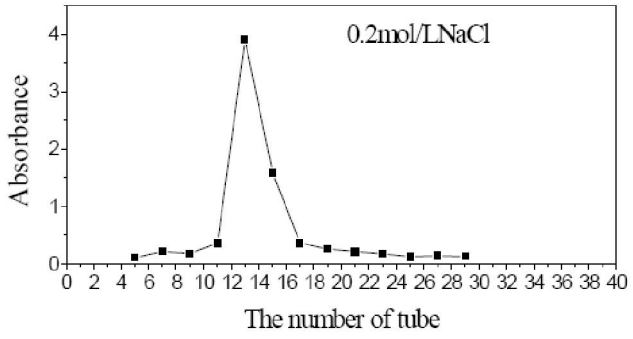

Figure 4 : Fractions eluted by 0.2 mol/L of NaCl.

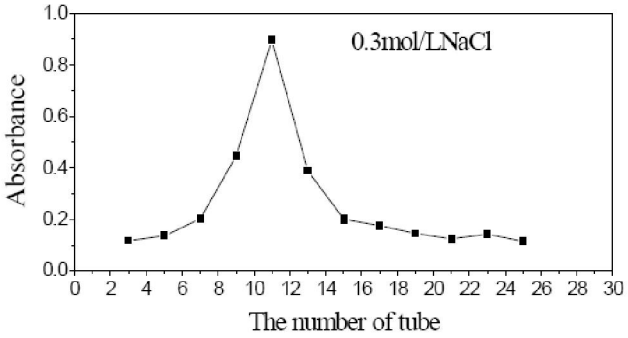

Figure 5 : Fractions eluted by 0.3 mol/L of NaCl.

freeze-dried product of the combination solution from tubes 7 to 23 using 0.1 mol/L of NaCl to rinse the column. Before freeze-drying, the combination solution had to be dialysed (MWCO 14000 Da) against distilled water first in order to remove NaCl. Dried $PsEUL1_2$ was obtained from tubes 11 to 17 under eluting by 0.2 mol/L of NaCl and $PsEUL1_3$ from tubes 7 to 15 at the condition of 0.3 mol/L of NaCl as eluent.

It was well known that the adsorbability of polysaccharides on DEAE-52 (OH) cellulose increased as the amount of acidic groups within polysaccharide molecule and their molecular weight increased. Therefore we assumed $PsEUL1_1$, $PsEUL1_2$ and $PsEUL1_3$ had different amount of uronic acid and molecular weight and sequently increased in their number of uronic acid and/or molecular weight.

To further investigate which polysaccharide in PsEUL1 group played the most important role in inhibiting activated complement, both of the classical pathway (CP) and the alternative pathway (AP) in which complement was activated were employed to evaluate bio-activities of $PsEUL1_1$, $PsEUL1_2$ and $PsEUL1_3$.

The TABLE 3 shows the three purified polysaccharides exhibited distinct differences in anti-complement activities in classical pathway. The inhibition rate of $PsEUL1_1$ was very low, whereas $PsEUL1_2$ and $PsEUL1_3$ possessed very high suppression of complement activity, especially $PsEUL1_3$. Although the inhibition rates of both $PsEUL1_2$ and $PsEUL1_3$ were little difference, the concentration of $PsEUL1_3$ for assay was only nearly one tenth of that of $PsEUL1_2$. According to their values of IC_{50} (50% inhibitory concentration, half inhibitory concentration), it could clearly demonstrate $PsEUL1_3$ possesses much higher inhibition than $PsEUL1_1$, and $PsEUL1_2$ as well. The IC_{50} of $PsEUL1_3$ (210 μg/mL) is less than one fifth of that of $PsEUL1_2$

(1080 μg/mL). $PsEUL1_2$ and $PsEUL1_3$ showed obvious dose-effect relationships.

TABLE 4 : The anti-complement activities of PsEULs in classical pathways.

Polysaccharide	Concentration (mg/mL)	Inhibition rate (%)	IC_{50} (mg/mL)
$PsEUL1_1$	3	23.76±4.16	-
	2	15.20±2.26	
	1	16.80±1.27	
$PsEUL1_2$	2.5	88.41±2.38	1.08
	1.5	71.20±0.38	
	1	48.64±4.16	
	0.75	31.72±4.49	
	0.5	8.11±0.27	
$PsEUL1_3$	0.3	86.80±2.07	0.21
	0.25	71.66±3.66	
	0.2	48.00±7.21	
	0.175	29.13±4.54	
	0.05	17.40±1.81	

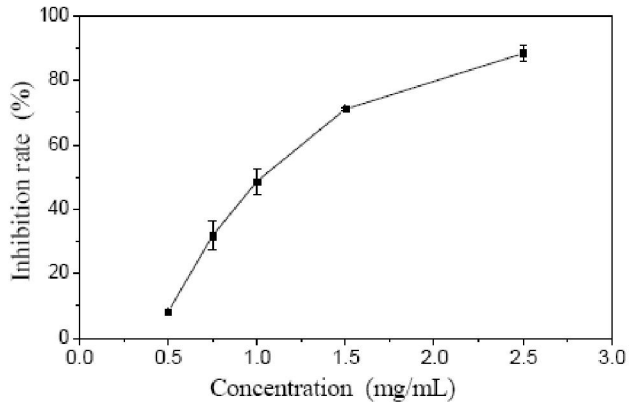

Figure 6 : The inhibition rate varied with the concentration of $PsEUL1_2$ in classical pathway.

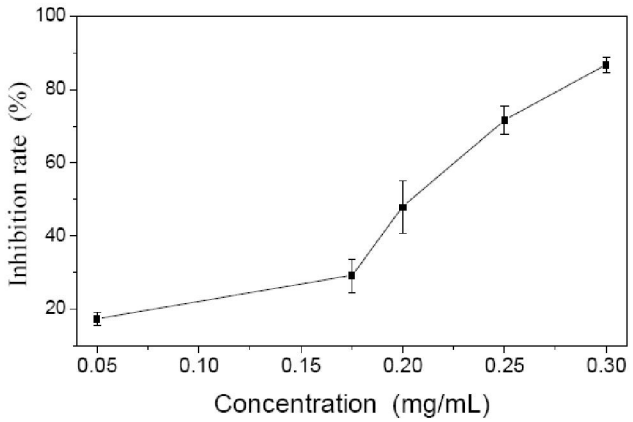

Figure 7 : The inhibition varied with the concentration of $PsEUL1_3$ in classical pathway.

Serum alternative pathway assay in altered form further demonstrated the results of classical pathway assay were correct, that is to say, $PsEUL1_3$ was the one that had the strangest inhibitory activity to activated complement among all obtained polysaccharides. Alternative pathway assay showed that $PsEUL1_1$ had no inhibitory activity and $PsEUL1_2$ exhibited an obvious inhibition only at a high concentration. Although with a very clear anti-complement activity and dose-effect relationship (see TABLE 5), $PsEUL1_3$ did not keep its inhibitory activity up with concentration but maintained at ~65% (see Figure 8). A slight positive variation from 0.5 mg/mL to 1.5 mg/mL implied, we supposed, $PsEUL1_3$ acted not only as enhancing but modulatory agent in inhibiting activated complement.

TABLE 5 : The anti-complement activities of PsEULs in alternative pathway.

Polysaccharide	Concentration (mg/mL)	Inhibition rate (%)
$PsEUL1_1$	3	0
	2	0
$PsEUL1_2$	3	77.08±2.38
	1.5	0
$PsEUL1_3$	1.5	67.80±2.00
	0.5	64.73±0.79
	0.2	27.98±1.05
	0.05	8.17±1.81

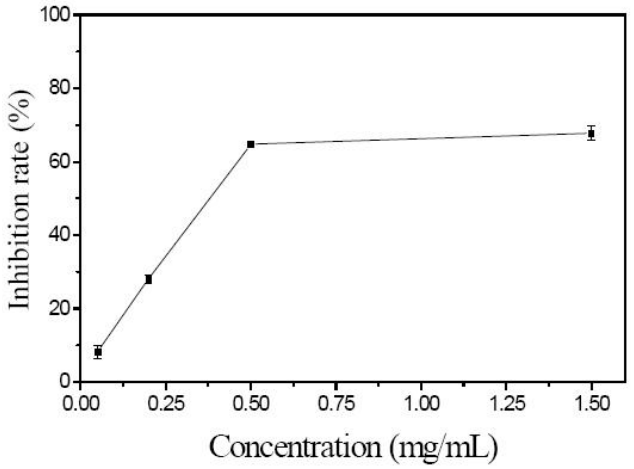

Figure 8 : The inhibitory activity varied with the concentration of $PsEUL1_3$ in alternative pathway.

Polysaccharide $PsEUL1_2$ was a powder with muddy colour and $PsEUL1_3$ a powder with yellowish brown. Both of them were insoluble in organic solvents, hardly soluble in cold water, slightly soluble in hot water and

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diluted acidic solution, but soluble in diluted alkaline solution, which demonstrated $PsEUL1_3$ and $PsEUL1_2$ are acidic polysaccharides and of higher molecular weight. In the investigation, it was found that the solubility of polysaccharides decreased evidently with the improvement of their purity. So the attempt to analyze their molecular weight by size exclusion chromatography failed because the purified polysaccharides did not dissolve in eluting solution. (who can solve this problem, please give a help to us).

Before the study of chemical structure, checks on the purities of $PsEUL1_2$ and $PsEUL1_3$ were carried out using different methods. The negative reaction of the polysaccharides with the Fehling reagent indicated that there was no free reduction sugar within $PsEUL1_2$ and $PsEUL1_3$. The reactions with iodine-potassium iodide were also negative, that is, no coloured matters produced, suggesting both polysaccharides were not the starch. As $PsEUL1_2$ and $PsEUL1_3$ mixed with ferric chloride solution no chromogenic reaction occurred, which meant the polysaccharides did not contain any phenol and phenolic substances, e.g. tannins. However, the Molish reaction was positive, which confirmed that $PsEUL1_2$ and $PsEUL1_3$ were sugars or polymers of sugars. Moreover, UV scanning of the purified polysaccharides from 190-400 nm was performed on a UV-2550 UV-visible spectrophotometer (Shimadzu Corporation, Jepen) to check whether they were glucoprotein or not. We could not see any the characteristic absorption peak of protein in Figure 9, which indicated that purified polysaccharides did not bind any proteins to their molecular chain.

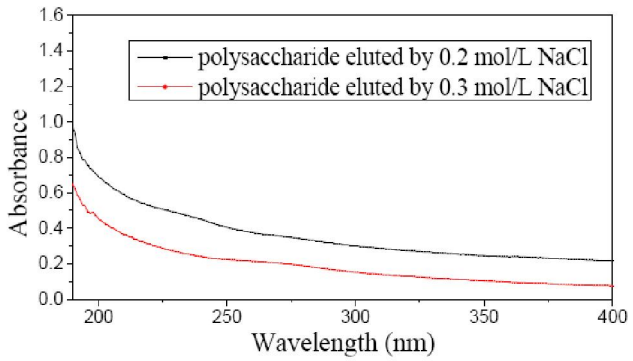

Figure 9 : UV absorbance of polysaccharides ($PsEUL1_2$ and $PsEUL1_3$) from *E. ulmoides* leaves from 190 nm to 400 nm.

According to our deduction above that $PsEUL1_3$ and $PsEUL1_2$ were acidic polysaccharides, obviously, the study of uronic acid was necessary. The measurement by the colorimetric method of sulfuric acid-carbazole[13] showed that $PsEUL1_2$ and $PsEUL1_3$ contain 43.8% and 21.2% of uronic acid, respectively (see TABLE 6). Standard material in the colourimetric determination was D-galacturonic acid (purchased from Sigma). Combined with the results of classical pathway and alternative pathway assays, we thought that the uronic acid presenting in polysaccharide played extremely vital role in inhabitation of activated complement. But a too high level of uronic acid within polysaccharide generated a negative effect on its inhabitation rate instead, such as $PsEUL1_2$ did.

TABLE 6 : The content of uronic (Galacturonic) acid of Eucommia leaf polysaccharide

Polysaccharide	Content of uronic acid (%)		
	test 1	test 2	mean
$PsEUL1_2$	42.17	45.46	43.8
$PsEUL1_3$	20.33	22.04	21.2

In addition to uronic acid, polysaccharide contained a great deal of non-acidic monosaccharides, which were analyzed by gas chromatography in this study. $PsEUL1_3$ was hydrolyzed to obtain monosaccharides and then, together with inositol (internal label), derived with acetic anhydride to form volatile species since monosaccharide was not volatile and could not be liberated in gas chromatographic condition. The formed volatile species were aldononitril derivativers that had to be reduced by hydroxylamine hydrochloride to form alditol before being derived by acetic anhydride. However, uronic acid could not be determined in gas chromatography for it could not be reduced by hydroxylamine hydrochloride and form volatile compound. The analysis

TABLE 7 : Monosaccharide compositions of polysaccharide $PsEUL1_3$.

Peak	Composition	Retaining time [min]	Peak area [uV*s]	Content [%]
1	L-rhamnose	16.744	6482	1.486
2	D-fucose	18.944	899	0.206
3	D-arabinose	20.576	20688	4.744
4	D-xylose	21.748	2290	0.525
5	D-glucose	35.465	5875	1.347
6	D-galactose	39.521	7051	1.617
7	inositol	41.411	392817	90.075
---	total	-------	436102	100.000

results are listed in TABLE 7. Qualitative analysis indicated that the monosaccharides of which $PsEUL1_3$ are composed are L-rhamnose, D-fucose, D-arabinose, D-xylose, D-glucoser and D-galactose.

Because uronic acid content in PsEUL13 was 21.2%, according to the normalization method, the relative percentages of six monosaccharides identified by gas chromatography should be further modified. The total relative percentages of six monisaccharides became 78.8% and the relative percentages of L-rhamnose, D-fucose, D-arabinose, D-xylose, D-glucose and D-galactose became 11.8%, 1.7%, 37.7%, 4.2%, 10.6% and 12.8%, respectively. It is must be noted that the relative percentages of these monosaccharides and uronic acid have some error because the determination method of uronic acid and monosaccharides was different. In addition, the further detailed structure of polysaccharide might be analyzed by optical rotation, methylation analysis, mass spectra and NMR spectra. But it was the poor dissoluble of the purified polysaccharides that deterred our attempt to analyze the structure of polysaccharide.

TABLE 8 : The relative content (percentage) of various glycosyl of $PsEUL1_3$.

Peak	Composition	Percentage in normalization (%)	Group content (%)	Relative percentage (%)
1	L-rhamnose	15.0		11.8
2	D-fucose	2.1		1.7
3	D-arabinose	47.8	78.8	37. 7
4	D-xylose	5.3		4.2
5	D-glucose	13.5		10.6
6	D-galactose	16.3		12.8
7	uronic acid		21.2	21.2
---	total	-------	100.000	100.000

From the discussion above, some conclusions could be made. It was found that the yield of polysaccharides (PsEUL1) precipitated by 50% solution of ethanol was the highest among three groups of polysaccharides precipitated by different concentration of ethanol, whereas protein content (0.8%) was lower than that of PsEUL2 (2.5%) and PsEUL3 (3.0%). Because of its strongest inhabitation, PsEUL1 was selected as the major object of this study and that the precipitation with various concentration of ethanol was adopted was due to that this precipitation method was very convenient for PsEUL1 separating and purifying. Decolourization is a very important process in the purification of extract fro leaf since leaves contain a great deal of tannins and chlorophyll. S-8 macroporous resins were chosen not only because of its highest decolourization but also because of its much higher retention rate of saccharide.

$PsEUL1_3$ polysaccharide from *E. ulmoides* Leaves had been demonstrated by both assayes of the classical pathway and alternative pathway to be a highly active inhibiter of actived complement. The polysaccharide is adcidic, which is very importent and essential for its inhibition, but an excessive acidic groups, or uronic acid, low its bio-acitivity instead.

EXPERIMENTAL SECTION

General experimental procedures

Some detection methods, such as Fehling reagent, phenol-sulfuric acid method[13], iodine-potassium iodide reaction, Molish reaction, ferric chloride reaction, and Coomassie brilliant blue colourimetric method[14], were applied to analyze the purities of polysaccharides and impurities within samples. Uronic acid contents in polysaccharide were determined using the colorimetric method of sulfuric acid-carbazole[15]. Polysaccharide samples and standard substance galacturonic acid were dissolved in distilled water as sample solutions and standard solution (100ug/mL), respectively. Place 1~2 mL such solutions in 10 mL colorimetric cylinders and add 5 mL of sodium tetraborate-sulfuric acid (0.478 g in 100 mL water) solution in each cylinder. Mix and heat the mixtures in boiling water for 20 min. When the mixtures were cooled to ambient temperature, 0.2 mL of 0.15% carbazole was used for coloration of uronic acid. After the coloration was going for 20 min, their optical densities were determined at 530 nm. A gas chromatograph GC-2014 and a SFAT capillary column (both are from Shimadzu Corporation, Jepen) coupled with hydrogen flame ionization detector was used to analysis the monose elements of which polysaccharides comprise[16]. Polysaccharide was hydrolyzed with trichloroacetic acid in nitrogen at 110°C and afterwards dried under reduced pressure. The hydrolyzate, or monoses, and inositol (internal label), before derived with acetic anhydride, was pre-reacted with hydroxylamine hy-

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drochloride in 5~9°C ice-water bath in the presence of pyridine. The obtained derivatives are aldononitrile acetates and, after airing at <70°C, dissolved in chloroform for analyzing by GC. The analysis was carried out from 180 to 250°C at 5°C/min, maintaining at 180°C for 1 min and at 250°C for 30min. Nitrogen was as carrier gas at 5 mL/min, and hydrogen at 40 mL/min and air at 450 mL/min were as combustion-supporting gas. The work temperature of FID was at 270°C.

Plant materials

Fresh Leaves of *E. ulmoides* were derived from *E. ulmoides* trees at Zunyi region of Guizhou Province, China, last in October 2009 and dried in the air at normal atmospheric temperature for following experiments. The temperature was chosen since it was sufficient to dry plant material without causing discoloration of the leaves. The plants were cultivated in their native environment and were at least 10 years old at time of harvest. The tree was very easy to identify because the plant is the unique one with a great deal of white guttapercha within its tissue and structure (bark and leaf), see Figure 1.

Extraction

Dried leaves of *E. ulmoides* Oliv were rubbed into small pieces (~3mm) by hands. For containing a large number of gutta percha the rubbed leaves were aggregated. Place rubbed leaves and a certain volume of petroleum ether (60~90ºC) in 1L three neck round bottom flask equipped with a comdenser for reflux. Stopper the unused openings in the flask and heat the mixture under reflux for 3h at 80 ºC under strongely magnetic stirring to remove resin and gutta percha. Use a water bath to heat. While the solution was still hot, filter it by gravity through a Buchner funnel using 100~140 mesh stainless steel screen. The residue (rubbed leaves) was extracted with distilled water under reflux in a 70 ºC hot water bath for 2h. Extraction was done three times. Filter them by vaccum through a Buchner funnel using a fast filter paper. The three extraction solutions were combined and evaporated at a temperature of <70 °C under reduced pressure to concentrate the combined solutions to specific gravity of ~1.2. Cool the concentrate to room temperature and precipitate carbohydrate by adding various volume of absolute alcohol, e.g. equal volume (containing 50% alcohol), double volume (67% alcohol) and triple volume (75% alcohol). Shake and centrifugalize the cloudy solution to obtain three group of carbohydrates, i.e. PsEUL1, PsEUL2 and PsEUL3.

Isolation

Aqueous solutions containing 2% (w/v) crude polysaccharides, PsEUL1, PsEUL2 and PsEUL3, were filtered using a fast filter paper to remove insoluble impurities and then applied to S-8 macroporous resin (Haiguang Chemical Co., Ltd, China) column to decolorize[17,18]. Deproteinize with Sevag method. Mix the Sevag reagent (chloroform:butanol = 5:1) with the decolored polysaccharide solution at 1:1. After shaked violently, the mixtures were centrifuged for 10min at 4000 rpm and the supernatants were collected and concentrated, repeating the operation 8 to 10 times until no precipitate at interface or no absorption peak at 278 nm. The decolored and deproteined polysaccharide solutions were dialyzed against water (Spectrapor MWCO 14000 Da), followed by freeze-drying to obtain treated polysaccharides[8]. Weigh 0.2g treated PsEUL1 and dissolve it in 3mL of distilled water. The solution was applied on DEAE-52 (OH) cellulose column (2.5cm × 25cm) coupled with a automatic fraction collector and rinsed with 0.0 → 1mol / L NaCl solution at 1mL/min. Each fraction was 10mL and the sulfuric acid - phenol method was used to track the eluted polysaccharides. Combine the fractions that belonged to an elution peak and obtain three polysaccharides, $PsEUL1_1$, $PsEUL1_2$, $PsEUL1_3$.

Anti-complement assay (the classical pathway (CP))[3,19]

Place 100μL of phosphate buffer saline (PBC) with or without sample (pH7.0 ~ 7.5) in test tubes, to which 100μL of normal guinea-pig serum (complement serum) diluted with glucose gelatin veronal buffered saline (GGVB) (1:100) and 100μL sensitized sheep red blood cells (5×108 cell/mL) were added. Shake tubes gently and the mixture was incubated at 37 °C as shaken gently from time to time for 30min. Then the incubation was terminated by adding 1mL of cold saline and the mixture were centrifuged at 4 °C, 2000rpm for 10min. Take the supernatant and measure the optical density

(OD) at 405nm. The calculation methods of the inhibiting rate of the samples against the hemolytic activity of complement as follows:

$$\text{Inhibition rate (\%)} = \frac{\text{Controls OD values} - \text{Sample OD value}}{\text{Controls OD values}} \times 100\%$$

Anti-complement assay (alternative pathway (AP))[19]

100μL of phosphate buffer saline (PBC) with or without sample (pH7.0 ~ 7.5) was placed in test tubes, to which an equal volume of GVB-Mg-EGTA buffer (containing 0.1% gelatin, 5mMC of veronal, 0.14mol/L of NaCl, 4mmol/L of MgCl2 and 16mmol/L of ethylene-glycol tetra-acetic acid, pH 7.4) dissolved with normal guinea-pig serum according to 2:1 and 100μL of rabbit red blood cell suspension (1.5×108 cell / mL) were added. Shake tubes gently and the mixture was incubated at 37 °C for 30min and shaken gently from time to time. Add 1mL of cold saline to terminate the incubation on time and centrifuge the mixture (4 °C, 2000 rpm) for 10min. The optical density of the supernatant was measured at 405nm. Calculate the inhibiting rate of the samples against the hemolytic activity of complement as above.

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SUPPORTING INFORMATION AVAILABLE

The pictures is available free via the Internet at http://www.eyoou.com.

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