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A protein-bound polysaccharide from the stem bark of *Eucommia ulmoides* and its anti-complementary effect

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ABSTRACT

Bioactivity-guided fractionation of a hot-water extract from the stem bark of *Eucommia ulmoides* led to the isolation of a homogeneous polysaccharide EWDS-2, which was identified as a highly branched protein-bound polysaccharide with average molecular weight between 1000 and 2000 kDa, composed of Glc, Gal, Ara, and Rha in the ratio of 2.2:1.0:0.4:0.2, along with traces of Man and 6.55% of protein. The main linkages of the residues of EWDS-2 include terminal, 1,3-linked, 1,4-linked, 1,2,6-linked, 1,3,6-linked Glc; 1,6-linked, 1,2,6-linked, 1,3,4-linked, 1,4,6-linked Gal; 1,5-linked, 1,3,5-linked Ara; terminal and 1,2,5-linked Rha. The bioassay revealed that EWDS-2 inhibits complement activation on both the classic and alternative pathways with CH_{50} and AP_{50} values of 282 ± 11 μg/mL and 144 ± 17 μg/mL, respectively. Preliminary mechanism studies indicate that EWDS-2 inhibits the activation of the complement system by interacting with C1q, C1r, C1s, C2, C3, C4, C5, and C9. The results suggested that EWDS-2 could be valuable for the treatment of diseases associated with the excessive activation of the complement system.

1. Introduction

Accumulating data have suggested that excessive activation of complement is involved in the pathogenesis of many auto-immune disorders, inflammatory diseases, and inflammation responses such as system lupus erythematosus, rheumatoid arthritis, and acute respiratory distress syndrome.[1–3] Therefore, inhibition of the complement system is one strategy in treating these diseases. Some natural polysaccharides including proteoglycans have been reported to have anti-complementary effects.[4–8] In our efforts to search for natural complement inhibitors from the traditional Chinese medicines, a hot-water extract of the stem bark of *Eucommia ulmoides* Oliv. (Eucommiaceae) was found to show strong anti-complementary activity from hemolytic assays, and further experiments revealed that the crude polysaccharides contributed much to the anti-complementary activity. Bioactivity-guided fractionation of the bioactive fraction led to the isolation of a protein-bound polysaccharide, EWDS-2, which was found to be an anti-complementary agent, in addition to the reported EWDS-1.[9] This paper describes the isolation and characterization of EWDS-2 and its anti-complementary properties.

2. Results

2.1. Structural characterization of EWDS-2

The yield of EWDS-2 from the stem bark of *E. ulmoides* was 2.3% of the dry material. EWDS-2 was obtained as a brown powder, $[\alpha]_D^{25}$ +99.3 (*c* 0.2, H_2O). The high-performance gel-permeation chromatography (HPGPC) (Fig. 1) and high-performance capillary electrophoresis (HPCE) (Fig. 2) profiles showed a single and nearly symmetrical peak, indicating that EWDS-2 was a homogeneous constituent, with an average molecular weight between 1000 and 2000 kDa.

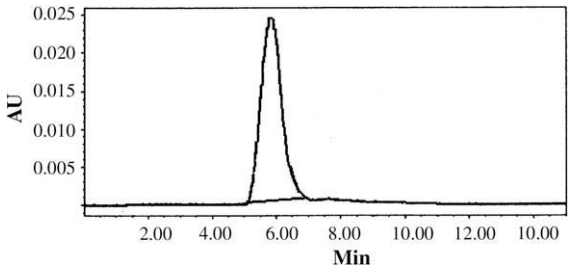

Figure 1. Profile of EWDS-2 in HPGPC. The sample was analyzed by a TSK-GEL GMPWXL gel-filtration column (7.6 mm × 300 mm, TOSOH) and eluted with 0.1 M NaCl at 0.8 mL/min.

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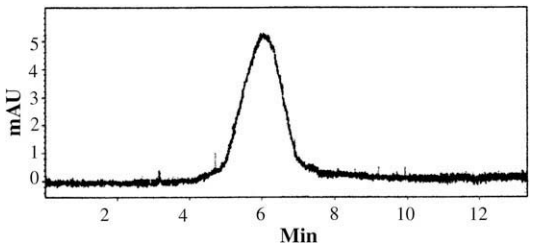

Figure 2. HPCE electropherogram of EWDS-2. The sample was analyzed by an uncoated fused-silica capillary tube (75 μm × 60 cm) using 0.01 M boric acid–NaOH buffer (pH 8.60) as solvent, with detection at 254 nm.

Elemental analysis revealed C, 30.96; H, 4.465; N, 1.5. The result of ultraviolet scanning showed that the maximum absorbance of EWDS-2 is around 210 nm, and the absorbance at 280 nm is very limited. The IR spectrum of EWDS-2 displayed a typical major broad stretching peak around 3423 cm^{-1} for the hydroxyl group, and a weak C–H stretching peak at 2915 cm^{-1}. However, IR peaks around 1700 cm^{-1}, which indicate the presence of uronic acid, and 1240 cm^{-1} for S═O, as well as 850 cm^{-1} and 820 cm^{-1} for C–O–S were not observed because of their small contribution to the mixture.[10] This was confirmed by an analysis that showed 3.45% uronic acid and 2.82% sulfate, as determined below. EWDS-2 contained 92.32% of total carbohydrate, as well as 6.55% of protein, which could not be removed by the Sevage reagent and trichloroacetic acid, but could be removed from EWDS-2 by the treatment with proteinase K (the protein content decreased from 6.55% to 0.1% according to Folin's phenol test).

The monosaccharide composition of EWDS-2 was determined by gas chromatographic analysis of the alditol acetates. The result showed that EWDS-2 contained glucose, galactose, arabinose, and rhamnose with a molar ratio of 2.2:1.0:0.4:0.2, along with a trace of mannose.

The ^{13}C NMR signal at δ 107.2 indicated that the configuration of the arabinose residue was α-L-Ara*f*, and the signals at δ 98.6–100.2 revealed the presence of α-D-Gal*p* and α-D-Glc*p*,[11] which was supported by IR band at 840 cm^{-1} and high positive optical rotation ($[\alpha]_D^{25}$ +99.3). The absence of a signal near δ 170, corresponding to the carboxyl group of uronic acid, indicated low percentage of uronic acid.

The sugar linkages of EWDS-2 were deduced from the methylation analysis (Table 1). Methylation analysis revealed terminal, 1,3-linked, 1,4-linked, 1,2,6-linked, 1,3,6-linked Glc; 1,6-linked, 1,2,6-linked, 1,3,4-linked, 1,4,6-linked Gal; 1,5-linked, 1,3,5-linked Ara; terminal and 1,2,5-linked Rha. The results revealed that the glucoses were mainly 1,4-linked and in the terminal positions, the galactoses were mainly 1,6-linked and 1,2,6-linked, the arabinoses were mainly 1,3,5-linked, and the rhamnoses were mainly in the terminal positions. The identification of large quantities of terminal, 1,2,6-linked Glc, 1,2,6-linked, 1,4,6-linked Gal, 1,2,5-linked, 1,3,5-linked Ara, and terminal Rha indicated that EWDS-2 was a highly branched polysaccharide (Table 1).

2.2. Inhibition of the complement system in vitro

The effect of EWDS-2 on the activation of human complement through the classical pathway was examined in 1:10 diluted normal human serum (NHS), with heparin serving as the positive control. The percentage of activation that 1:10 diluted NHS occurred in the classic pathway was 97.3 ± 5.2% in the complement control group. The concentrations that resulted in 50% inhibition (CH_{50}) were 282 ± 11 μg/mL and 85 ± 30 μg/mL for EWDS-2 and heparin, respectively, as shown in Figure 3. They both blocked hemolysis of sensitized erythrocytes (EAs) in a dose-dependent manner. EWDS-2 was weaker than heparin in inhibiting activation of the classical pathway, although it almost abolished all of the hemolytic activity of NHS (1:10) (percent inhibition: 94.3 ± 6.5%) at a concentration of 650 μg/mL.

The percentage of activation that 1:10 diluted NHS showed in the alternative pathway was 96.2 ± 7.7% in the complement control group. As shown in Figure 4, the concentrations that resulted in 50% hemolysis inhibition (AP_{50}) of rabbit erythrocytes (ERs) on the alternative pathway were 144 ± 17 μg/mL for EWDS-2 and 102 ± 22 μg/mL for heparin, respectively. EWDS-2 was a little

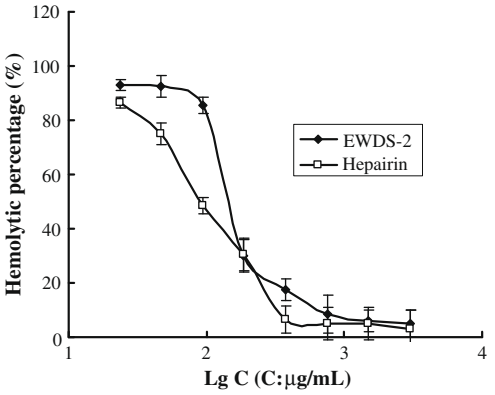

Figure 3. Inhibition of classical pathway-mediated hemolysis of EAs in 1:10 diluted NHS in the presence of increasing amounts of EWDS-2 (◆). Heparin (□) was used as reference. Results are expressed as hemolytic percentage. Data are the mean values from 4 determinations ± SEM.

Table 1
Alditol acetate derivatives from the methylated EWDS-2

Methylated sugars (as alditol acetates)	Type of linkage	Molar ratio	Mass fragments (*m/z*)
2,3,4,6-Me_4-Glc	Terminal Glc*p*	21.44	43, 71, 87, 101, 117, 129, 145, 161, 205
2,4,6-Me_3-Glc	1,3-Linked Glc*p*	3.44	43, 58, 71, 87, 99, 101, 117, 129, 161, 201, 233
2,3,6-Me_3-Glc	1,4-Linked Glc*p*	25.96	43, 45, 71, 87, 99, 101, 113, 117, 129, 161, 233
2,4-Me_2-Glc	1,3,6-Linked Glc*p*	0.78	43, 87, 99, 101, 117, 129, 189, 201, 233
3,4-Me_2-Glc	1,2,6-Linked Glc*p*	2.12	43, 87, 99, 129, 189, 233
2,3,4-Me_3-Gal	1,6-Linked Gal*p*	13.73	43, 45, 71, 87, 99, 101, 117, 129, 161, 189, 233
2,6-Me_2-Gal	1,3,4-Linked Gal*p*	0.97	43, 57, 87, 117, 129, 161, 143, 203, 233
2,3-Me_2-Gal	1,4,6-Linked Gal*p*	1.82	43, 57, 87, 99, 101, 129, 159, 187, 201
3,3-Me_2-Gal	1,2,6-Linked Gal*p*	5.95	43, 87, 99, 129, 159, 189, 233
2,3-Me_2-Ara	1,5-Linked Ara*f*	1.73	43, 71, 87, 99, 101, 117, 129, 189
2-Me-Ara	1,3,5-Linked Ara*f*	7.12	43, 57, 87, 117, 129, 161, 189
3-Me-Rha	1,2,5-Linked Rha*p*	1.27	59, 71, 85, 87, 99, 129, 159, 189
2,3,5-Me_3-Rha	Terminal Rha*p*	3.62	71, 87, 101, 117, 129, 145, 161

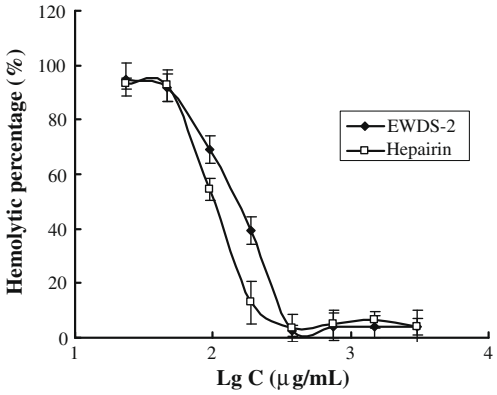

Figure 4. Inhibition of alternative pathway-mediated lysis of ERs in 1:10 diluted NHS in the presence of increasing amounts of EWDS-2 (◆). Heparin (□) was used as reference. Results are expressed as hemolytic percentage. Data are the mean values from 4 determinations ± SEM.

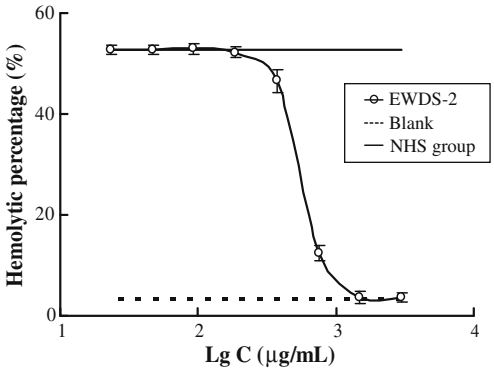

Figure 5. Hemolytic activity of SRBC in 1:4 diluted NHS in the presence of increasing amounts of EWDS-2 (○). Hemolytic activity of the blank group containing SRBC in buffer (- - -) and the NHS group containing NHS and SRBC in buffer (—) were used as references.

weaker than heparin in inhibiting activation through the alternative pathway.

The protein-free EWDS-2 showed the similar anti-complementary effects (CH_{50}: 280 ± 19 μg/mL; AP_{50}: 136 ± 14 μg/mL) as the original EWDS-2, indicating that the existence of protein in EWDS-2 did not affect its anti-complementary activity.

2.3. Confirming the inhibiting effect of EWDS-2 on the complement

The data obtained from the assays without pre-incubation showed that EWDS-2 (CH_{50}: 306 ± 26 μg/mL; AP_{50}: 181 ± 18 μg/mL) and heparin (CH_{50}: 108 ± 15 μg/mL; AP_{50}: 122 ± 13 μg/mL) still exhibited an inhibitory effect without pre-incubation; however, the activity potency of EWDS-2 and heparin both decreased markedly, as compared to that obtained with a pre-incubation of 10 min. This result indicated that EWDS-2 acted on complement similarly as heparin and should be an anti-complementary agent.

The 1:4 diluted NHS gave 52.67 ± 3.65% of hemolysis after incubation with SRBC (sheep red blood cell) at 37 °C for 30 min and was chosen to examine the effect of EWDS-2 on spontaneous activation of NHS. The results (Fig. 5) showed that at concentrations of EWDS-2 higher than 1000 μg/mL, the hemolysis (3.35 ± 1.02%) was as low as that of the blank group (3.29 ± 0.16%); at concentrations of EWDS-2 lower than 1000 μg/mL, the hemolysis increased with the decrease of concentrations of EWDS-2 and reached to 52.73 ± 2.05% at the concentration of EWDS-2 lower than 93 μg/mL. This observation confirmed the inhibitory effect of EWDS-2 on spontaneous activation of NHS.

2.4. Identification of the target individual complement of EWDS-2 in the complement activation cascade

The effects of EWDS-2 on individual complement components were investigated in the system with complement-depleted reagents and a limited amount of human serum. The capacities of various depleted sera to restore the hemolytic capacity of EWDS-2-treated serum were examined. Under these conditions, the complement component under investigation is the limiting factor in the component-mediated hemolysis assay. Thus, the failure of restoring the hemolysis could be attributed to the interaction between the tested sample and corresponding complement component.

As shown in Figure 6, the percentage of the 1:10 NHS-induced hemolysis through the classical pathway was 96.4 ± 9.1% in the complement control group. EWDS-2 at a concentration of 650 μg/mL exhibited a strong inhibitory effect on this hemolysis; its hemolysis percentage was 8.6 ± 2.3%. None of the C-depleted sera lyse EAs independently (including C1q, C1r, C1s, C2, C3, C4, C5, and C9). The hemolysis percentages of the target groups were no more than 10%, indicating that addition of complement-depleted serum did not restore any hemolytic activity of EWDS-2-treated sera. Therefore, EWDS-2 inhibits complement activation by blocking all the target complements used in this assay.

2.5. Influence of EWDS-2 on coagulation system

From the data of coagulation assays (Table 2), EWDS-2 almost had no effect on recalcification time (RT) and thrombin time (TT). For instance, at the concentration of 5.2 μg/mL, heparin markedly

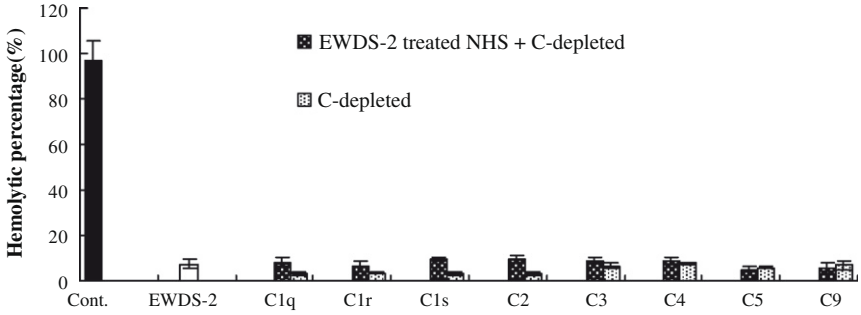

Figure 6. Hemolytic assays for individual components utilizing C-depleted serum. EWDS-2-treated serum was mixed with various depleted sera, and the capacity of these depleted sera to restore classical pathway hemolytic capacity was estimated by adding sheep antibody-sensitized erythrocytes. Results are expressed as hemolytic percentages. Data are the mean values from 4 determinations ± SEM.

Table 2
Effects of EWDS-2 on coagulation system ($\bar{x} \pm SD$), $n = 6$

Sample	Vehicle	Heparin	EWDS-2		
	—	5.2 (mg/L)	1500 (mg/L)	750 (mg/L)	375 (mg/L)
RT(s)	60.4 ± 2.2	136.5 ± 3.7*	60.8 ± 1.3	60.1 ± 2.2	61.5 ± 1.8
TT(s)	91.0 ± 1.8	268.1 ± 2.9*	94.9 ± 1.4	90.9 ± 1.4	92.9 ± 1.0

* $P < 0.05$, significantly different from the vehicle.

prolonged recalcification time (RT: 136.5 ± 3.7 s) and thrombin time (TT: 268.1 ± 2.9 s) in contrast with the vehicle control (RT: 60.0 ± 2.2 s, TT: 91.0 ± 1.8 s), whereas the clotting time for EWDS-2 on RT (60.8 ± 1.3 s) and TT (94.9 ± 1.4 s) showed no difference with the vehicle control even at a higher concentration (1.5 mg/mL).

3. Discussion

Numerous natural carbohydrate polymers have been reported to possess anti-complementary activity based on the commonly used method of hemolytic assays. However, polysaccharides have also been identified as complement activators.[12,13] In order to determine whether the activating effect was involved in EWDS-2, we have performed hemolytic assays without pre-incubation (not involved in the activating mechanism) and assays on observing the effect of EWDS-2 on spontaneously activated NHS. The results showed that EWDS-2 acted on complement in a fashion similar to that of heparin in the assays without pre-incubation, indicating that EWDS-2 should be an inhibitor of complement. In the assays on observing the effect of EWDS-2 on spontaneously activated NHS, as the concentration of EWDS-2 increased, the decreased hemolysis percentages from the level of the NHS group to that of the blank group further confirmed the inhibitory effect of EWDS-2.

For anti-complementary activity of carbohydrate polymers, the sulfate group has been found to be a requirement.[14,15] High arabinose and galactose content,[4,16–19] as well as the existence of branched structures,[5–7] has also been considered important to the anti-complementary activity of polysaccharides and glycoproteins. The anti-complementary EWDS-2 was characterized with a branched structure composed of Ara and Gal in high concentration, with 2.82% sulfate content, which supports the above-mentioned viewpoints. In addition, our study revealed that the existence of protein did not affect the anti-complementary activity of protein-bound polysaccharides.[9]

The stem bark of *E. ulmoides*, known as 'Du-Zhong', has long been used in traditional Chinese medicine for the treatment of hypertension, rheumatic arthritis, lumbago, and ischialgia.[20–22] It was found to possess anti-inflammatory and immunological activities,[23–25] which are closely related to the complement system.[26–28] Although genipin and lignan glycosides from *E. ulmoides* have been reported to possess anti-complementary activity,[29] our investigation indicated that polysaccharides should be also important anti-complementary constituents in the water extract of *E. ulmoides*.

The natural sulfated polysaccharides (fucans) from brown seaweed have been reported to inhibit C1 activation or C4 cleavage.[30] In our previous study, D3–S1, a polysaccharide isolated from a *Bupleurum* medicinal plant, was found to selectively block activation of C1s, C3, and C4.[8] The present study showed that EWDS-2 blocked all the activation of C1q, C1r, C1s, C2, C3, C4, C5, and C9. It is obvious that these polysaccharides have different mechanisms in inhibiting the complement system, although they share some similarity on the molecular weight and sugar composition. In fact, the inhibitory mechanism of EWDS-2 on the complement system was much like that of heparin. However, heparin has been reported to interfere with the complement system at multiple levels, but is limited in clinical use on account of its anticoagulant properties.[31] Data from the current study demonstrates that EWDS-2, with extensive inhibitory targets on complement activation, but no effects on coagulation system, has considerable value over heparin in complement inhibition. The results suggest that EWDS-2 could be valuable for the treatment of diseases associated with the excessive activation of complement system. However, more comprehensive experiments to explore the implication of EWDS-2 on animal models are required.

4. Experimental

4.1. General methods

The stem bark of *E. ulmoides* was purchased from Huayu Materia Medica Co., Ltd, Shanghai, in October 2005. The plant material was verified by Dr. Daofeng Chen, and a voucher specimen (DFC-DZ2005101301) has been deposited at the Herbarium of Materia Medica, Department of Pharmacognosy, School of Pharmacy, Fudan University, Shanghai, PR China. Sephacryl® S-400 High Resolution and DEAE Sepharose® Fast Flow were purchased from Amersham Biosciences. Trifluoroacetic acid (TFA) was purchased from Fluka. All other reagents were of the highest available quality.

Sheep erythrocytes were collected in Alsevers' solution. Anti-sheep erythrocyte antibody was from rabbit antiserum, provided by Prof. Yunyi Zhang (Department of Pharmacology, School of Pharmacy, Fudan University, Shanghai, PR China). Rabbit erythrocytes were obtained from the ear vein of New Zealand white rabbits. NHS was obtained from healthy male donors (average age of 20 years). Guinea pigs were purchased from Laboratory Animals Research Institute of Fudan University (Shanghai, China). Heparin (sodium salt, 160 IU/mg) was from Shanghai Aizite Biotech Co. Ltd. Thrombin (5 U/mL) was purchased from Shanghai Yingyue Biological Co. Ltd. Anti-C1q, Human (Goat); Anti-C2, Human (Goat) were from E. Merck Biosciences; Anti-C5a, Human (Rabbit) were from Shanghai Shensuo Reagent Co. Ltd and Anti-C9, Human (Goat) were from Calbiochem. Anti-C1r, Human (Goat) and Anti-C1s, Human (Goat) were from Abcam. Anti-C3, Human (Goat) and Anti-C4, Human (Goat) were from Shanghai Sun Biotech Co. Ltd. Buffers: isotonic veronal-buffered saline (VBS^{2+}), containing 0.5 mM Mg^{2+} and 0.15 mM Ca^{2+}. EGTA–VB: veronal buffer saline, containing 5 mM Mg^{2+} and 8 mM EGTA (ethyleneglycol bis(2-aminoethyl ether)tetraacetic acid). The optical rotation was determined at 25 °C with a JASCO P-1020 polarimeter. The infrared spectrum (KBr pellets) was recorded on an FTIR spectrophotometer (Avatar 360 ESP, Thermo–Nicolet, USA). The UV spectrum was recorded on a UV–vis spectrophotometer (UNICO UV-2000). NMR spectrum was recorded with a DRX 400 spectrometer (Bruker Co. Ltd, Switzerland) in D_2O. Elemental analysis (C, H, and N) was conducted with a Perkin–Elmer 2400 instrument. The total carbohydrate content was determined by phenol–sulfuric acid,[32] using D-glucose, D-galactose, L-arabinose, and L-rhamnose (molar ratio: 2.2:1.0:0.4:0.2) as standards. Uronic acid content was determined by the *m*-hydroxybiphenyl method with D-galacturonic acid as the standard.[33] The concentration of total protein was estimated by the Folin phenol method of Lowry et al.,[34] using bovine serum albumin as standard. The sulfate content of the polysaccharide was measured using a modification of $BaCl_2$ turbidimetric method described by Craige et al.[35] Dialysis was carried out by using dialysis tubing (Spectra/Por MWCO: 6000–8000). Proteinase K (38.6 U/mg) was from Ameresco.

4.2. Extraction and purification of EWDS-2

The hemolytic assay on the classical pathway was used as the biological activity guide in fractionation of the anti-complemen-

tary principles from the stem bark of *E. ulmoides*. The bark (9 kg) of *E. ulmoides* was pulverized into powder and defatted with 95% EtOH, and the insoluble portion was extracted with hot water. The proteins in the hot-water extract were precipitated with trichloroacetic acid. The resulting aqueous fraction was extensively dialyzed against running water for 3 days, and the polysaccharide was precipitated by adding 4 volumes of 95% EtOH. After centrifugation, the precipitate was washed with anhyd EtOH and then freeze-dried to yield the crude polysaccharide. Among the EtOH extract, the water extract, and the crude polysaccharide, the strongest anti-complementary activity was observed in the crude polysaccharide fraction. The crude polysaccharide was further fractionated on a DEAE Sepharose® Fast Flow column by eluting with water, followed by stepwise increasing concentrations of NaCl solution (0.4, 0.8, 1.2, and 2.0 M NaCl), leading to the isolation of five sub-fractions, E1, E2, E3, E4, and E5. Of those, sub-fractions E3–E5 showed relatively high activity. Therefore the E3–E5 sub-fractions were combined and subjected to repeated size-exclusion column chromatography on Sephacryl® S-400 to give EWDS-2, which displayed greater anti-complementary activity.

4.3. Homogeneity and molecular weight

The homogeneity was determined by HPGPC and HPCE. The molecular weight of EWDS-2 was determined by HPGPC analysis. HPGPC was analyzed with a TSK GMPWXL gel-filtration column (7.6 mm × 300 mm, TOSOH) and eluted with 0.1 M NaCl at 0.8 mL/min. Commercially available standard dextrans T-2000, T-500, T-70, T-40, and T-10 were used as standard molecular markers. HPCE was performed on an uncoated fused-silica capillary tube (75 μm × 60 cm) at 25 °C using 0.01 M boric acid–NaOH buffer (pH 8.6) as solvent, with detection at 254 nm.

4.4. Sugar composition analysis and linkage analysis

EWDS-2 (7.0 mg) was hydrolyzed with 2 M TFA (trifluoroacetic acid) at 110 °C for 4 h. After removal of the acid and reduction with 20 mg of $NaBH_4$, the monosaccharide alditol acetates were prepared using the method described by Jones and Albersheim.[36] The alditol acetates were subjected to GC analysis on an HP 6890 GC (Hewlett–Packard, Wilmington, DE, USA) fitted with a capillary column DB-225 (0.25 mm × 30 m).

The linkage of EWDS-2 was analyzed from GC–MS results of partially methylated alditol acetates. The methylation reaction was performed according to the procedures of Ciucanu and Kerek.[37] Briefly, EWDS-2 (12.0 mg) was dissolved in DMSO under nitrogen and permethylated by treatment with 20.0 mg of NaOH powder and 0.5 mL of iodomethane. Partially methylated alditol acetates were prepared from the fully methylated sample by acid hydrolysis with 2 M TFA at 110 °C for 1 h. After reduction of the hydrolysates with $NaBH_4$ and acetylation of the alditols with Ac_2O, the alditol acetates were analyzed by GC–MS on the HP 6890 GC equipped with an HP 5973 mass-selective detector.

4.5. Preparation of the protein-free EWDS-2

EWDS-2 (10 mg) was dissolved in 5 mL H_2O, then treated with the Sevage reagent (4:1 CH_3Cl–*n*-BuOH, 20 mL × 7) and trichloroacetic acid (terminal concentration of trichloroacetic acid was 6% under cold conditions). After centrifugation, the supernatant was neutralized with 1 M NaOH, then dialyzed and freeze-dried for the measurement of protein content.

EWDS-2 (10 mg) was dissolved in 5 mL of 10 mM Tris–HCl buffer (pH 7.8) containing 5 mM EDTA (ethylenediamine tetraacetic acid) and 0.5% SDS (sodium dodecylsulfate), and 5 mL of a solution containing proteinase K (2 mg) in the same buffer was added.[38] After incubation at 37 °C for 2 h, the reaction was terminated by boiling for 0.5 h. The mixture was centrifuged, and the supernatant was freeze-dried after dialysis and assayed for protein content and anti-complementary activity in vitro.

4.6. Anti-complementary activity in vitro

4.6.1. Anti-complementary activity through the classical pathway

Based on Mayers' modified method,[39] sensitized erythrocytes (EAs) were prepared by incubation of sheep erythrocytes (4.0×10^8 cells/mL) with an equal volume of rabbit anti-sheep erythrocyte antibody in VBS^{2+}. EWDS-2 and heparin, used as the positive control, were dissolved in VBS^{2+}. NHS was used as the complement source. The 1:10 diluted NHS was chosen to give submaximal lysis in the absence of complement inhibitors. In brief, various dilutions of tested samples (100 μL) were pre-incubated with 100 μL NHS and 200 μL VBS^{2+} at 37 °C for 10 min. Then, 200 μL of EAs was added, and the mixture was incubated at 37 °C for 30 min. The different assay controls were incubated under the same conditions: vehicle control (200 μL EAs in 400 μL VBS^{2+}); 100% lysis (200 μL EAs in 400 μL water); sample control (100 μL dilution of each sample in 500 μL VBS^{2+}). The reaction mixture was centrifuged immediately. Optical density of the supernatant was measured at 405 nm with a spectrophotometer (Wellscan MK3, Labsystems Dragon). The absorbance of the control sample was subtracted from each value to obtain a corrected absorbance, and the percent inhibition was calculated.

4.6.2. Anti-complementary activity through the alternative pathway

According to the method of Klerx et al.,[40] each sample was dissolved in EGTA-VB, and various dilutions were prepared. After pre-incubation of the dilutions of each sample (150 μL) with 1:10 diluted NHS (150 μL) at 37 °C for 10 min, rabbit erythrocytes (200 μL, ERs 3.0×10^8 cells/mL) were added. Following a second incubation step at 37 °C for 30 min, cell lysis was determined as described in Section 4.6.1. Controls for vehicle, 100% lysis, and sample were included.

4.6.3. Confirming the inhibitory effect of EWDS-2 on the complement

The assays in Sections 4.6.1 and 4.6.2 were repeated without pre-incubation before addition of EAs or ERs. The 1:4 diluted NHS was chosen from a series of NHS dilutions (1:2; 1:4; 1:8; 1:10; 1:16; 1:20; 1:40; 1:80) to observe the effect of EWDS-2 on spontaneous activation of NHS with SRBC without pre-incubation. EWDS-2 (200 μL, as stepwise diluted concentrations ranging from 23.4 μg/mL to 3000 μg/mL) was diluted with 100 μL VBS^{2+}, then 100 μL of SRBC and 200 μL of NHS (1:4) were added. This mixture was incubated at 37 °C for 30 min. Controls include: 200 μL EWDS-2 (stepwise diluted concentrations) in 300 μL of VBS^{2+} and 100 μL of SRBC; NHS group (200 μL NHS in 300 μL of VBS^{2+} and 100 μL of SRBC); 100% hemolysis (100 μL of SRBC in 500 μL of water); blank (100 μL of SRBC in 500 μL of VBS^{2+}); sample control (200 μL of EWDS-2 (stepwise diluted concentrations) in 400 μL of VBS^{2+}). The reaction mixture was centrifuged, the optical density of the supernatant was measured, and the percent hemolysis was calculated.

4.7. Preparation of complement-depleted serum with complement antibody

This experiment was conducted according to the modified method of Zhou et al.[41] Various dilutions of each antiserum were incubated with the same volume of NHS (1:10) at 37 °C for

15 min. After centrifugation, supernatant (200 μL) was incubated with 200 μL of VBS^{2+} and 200 μL of EAs, then cell lysis was measured as described in Section 4.6.1. The antiserum dilution against the NHS hemolytic capacity was then determined. The optimal dilutions (1:32 for C1r, C1s, C5; 1:1 for C3, C4, and 1:64 for C1q, C2, C9) were incubated with the same volume of NHS (1:10) at 37 °C for 15 min, followed by centrifugation. The supernatants were stored as C-depleted sera in aliquots at −70 °C until they were used in the hemolytic assays.

4.8. Interaction with individual complement components

The capacity of depleted sera to lyse EAs through the classical pathway was assessed in the presence or absence of EWDS-2-treated NHS. EWDS-2-treated NHS was obtained by incubating optimally diluted EWDS-2 (650 μg/mL) with an equal volume of 1:10 diluted NHS at 37 °C for 10 min. The concentration of EWDS-2 just sufficient to cause complete loss of hemolytic activity of a 1:10 diluted NHS was determined in the section of anti-complementary activity through the classical pathway. For the target complement group (the assay for the capacity of various depleted sera to restore the hemolytic capacity of EWDS-2-treated serum), EAs (200 μL) and individual depleted serum (200 μL) of C1q, C1r, C1s, C2, C3, C4, C5, or C9 were added to 200 μL of EWDS-2-treated NHS, and the mixture was incubated at 37 °C for 30 min. After centrifugation and measurement of the optical density of the supernatant, the percent hemolysis was calculated. For the assay of an individual depleted serum group, C-depleted sera were directly incubated with EAs under the same conditions, and the hemolytic activities were calculated. Vehicle control (200 μL of EAs in 400 μL of VBS^{2+}), 100% lysis (200 μL of EAs in 400 μL of water), complement control (100 μL of NHS (1:10) and 200 μL of EAs in 300 μL of VBS^{2+}), EWDS-2 (200 μL of EWDS-2-treated NHS and 200 μL of EAs in 200 μL of VBS^{2+}), and sample control (100 μL of sample in 500 μL of VBS^{2+}) were incubated under the same conditions.

4.9. Influence on recalcification time (RT) and thrombin time (TT)

Platelet-poor plasma (PPP, obtained from blood of major arteries of Guinea pigs, 150 μL) was added into 15 μL of different concentration of EWDS-2 (1500 mg/L, 750 mg/L, and 375 mg/L, diluted with VBS^{2+}) or heparin (5.2 mg/L, used as the positive control), or VBS^{2+} (used as the vehicle control). The mixture was incubated at 37 °C for 5 min, then 150 μL of 0.025 M $CaCl_2$ solution was added. The time from the addition of $CaCl_2$ to clot formation was recorded as plasma recalcification clotting time, briefly called recalcification time (RT). The determination of thrombin time (TT) was identical to the determination of RT, except that $CaCl_2$ was substituted with thrombin.

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References

1. Makridea, S. C.. *Pharmacol. Rev.* **1998**, *50*, 59–87.
2. Lustep, H. L.; Clark, W. M. *Curr. Neurol. Neurosci. Rep.* **2001**, *1*, 13–18.
3. Peiris, J. S. M.; Chu, C. M.; Cheng, V. C. C.; Chan, K. S.; Hung, I. F. N.; Poon, L. L. M.; Law, K. I.; Tang, B. S. F.; Hon, T. Y. W.; Chan, C. S.; Chan, K. H.; Ng, J. S. C.; Zheng, B. J.; Ng, W. L.; Lai, R. W. M.; Guan, Y.; Yuen, K. Y.and members of the HKU/UCH SARS Study Group. *Lancet* **2003**, *361*, 1767–1772.
4. Wagner, H.; Stuppner, H.; Puhlmann, J.; Brümmer, B.; Deppe, K.; Zenk, M. H. *Z. Phytother.* **1989**, *10*, 35–38.
5. Yamada, H.; Nagai, T.; Cyong, J. C.; Otsuka, Y.; Tomoda, M.; Shimizu, N.; Shimada, K. *Carbohydr. Res.* **1985**, *144*, 101–111.
6. Zhao, Q. C.; Kiyohara, H.; Yamada, H. *Phytochemistry* **1994**, *35*, 73–77.
7. Kiyohara, H.; Zhang, Y.; Yamada, H. *Carbohydr. Polym.* **1997**, *32*, 249–253.
8. Xu, H.; Zhang, Y. Y.; Zhang, J. W.; Chen, D. F. *Int. Immunopharmacol.* **2007**, *7*, 175–182.
9. Zhu, H. W.; Zhang, Y. Y.; Zhang, J. W.; Chen, D. F. *Int. Immunopharmacol.* **2008**, *8*, 1222–1230.
10. Stancioff, D. J.; Stanley, N. F. *Proc. Int. Seaweed Symp.* **1969**, *6*, 595–609.
11. Sun, H. H.; Wooten, J. B.; Ryan, W. S.; Bokelman, J. G. H.; Aman, P. *Carbohydr. Polym.* **1987**, *7*, 143–158.
12. Alban, S.; Classen, B.; Brunner, G.; Blaschek, W. *Planta Med.* **2002**, *68*, 1118–1124.
13. Tatsunori, Y.; Yamaguchi, A.; Saito, T.; Seguchi, M. *J. Sci. Food Agric.* **2008**, *88*, 805–812.
14. Maillet, F.; Petitou, M.; Choay, J.; Kazatchkine, M. D. *Mol. Immunol.* **1988**, *25*, 917–923.
15. Kazatchkine, M. D.; Fearon, D. T.; Metcalfe, D. D.; Rosenberg, R. D.; Austen, K. *J. Clin. Invest.* **1981**, *67*, 223–228.
16. Beuscher, N.; Bodinet, C.; Willigmann, I.; Egert, D. *Z. Phytother.* **1995**, *16*, 157–166.
17. Lohmann-Matthes, M. L.; Wagner, H. *Z. Phytother.* **1989**, *10*, 52–59.
18. Thude, S.; Classen, B. *Phytochemistry* **2005**, *66*, 1026–1032.
19. Benencia, F.; Rodriguez, M. C.; Matulewicz, C.; Coulombié, F. C. *Phytochemistry* **1999**, *50*, 57–62.
20. Xie, W. Z.; Fan, C. S.; Zhu, Z. Y. *Quan Guo Zhong Cao Yao Hui Bian*; People's Medical Publishing House: Beijing, 1996. pp 423–424.
21. Ge, J. R.; Wang, H. M.; Yang, L. Z.; Yin, H. B.; Feng, X. H.; Jiang, Q.; Zhu, L. G.; Zhang, J.; Li, J. X.; Guo, Z. J.; Luo, T.; Huang, M. H. *Chinese J. Trad. Med. Traum. Orthop.* **2002**, *10*, 19–23.
22. Jiangsu New Medical College. In *Dictionary of Chinese Herbal Medicine*; Shanghai Science and Technology Publishing House: Shanghai, 1977; Vol. 1. pp 1031–1033.
23. Deyama, T.; Nishibe, S.; Nakazawa, Y. *Acta Pharmacol. Sinica* **2001**, *22*, 1057–1070.
24. Tomada, M.; Gonda, R.; Shimizu, N.; Kanari, M. *Phytochemistry* **1990**, *29*, 3091–3094.
25. Xu, S. L.; Zhou, H. Q.; Huang, W. G.; Zeng, Q. Z.; Liu, M.; Pan, Z. X. *Chin. Tradit. Herb Drugs* **1983**, *14*, 27–28.
26. Abe, M. *Jpn. J. Clin. Pathol.* **2006**, *54*, 744–756.
27. Sahu, A.; Lambris, J. D. *Immunopharmacology* **2000**, *49*, 133–148.
28. Sarma, V. J.; Huber-Lang, M.; Ward, P. A. *Autoimmunity* **2006**, *39*, 387–394.
29. Oshima, Y.; Takata, S.; Hikino, H.; Deyama, T.; Kinoshita, G. *J. Enthnopharmacol.* **1988**, *23*, 159–164.
30. Sahu, A.; Pangbrn, M. K. *Mol. Immunol.* **1993**, *30*, 679–685.
31. Blondin, C.; Fischer, E.; Boisson-Vidal, C.; Kazatchkine, M. D.; Jozefonvicz, J. *Mol. Immunol.* **1994**, *31*, 247–253.
32. Dubios, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. *Anal. Chem.* **1956**, *28*, 250–256.
33. Blumenkranz, N.; Asboe-hansen, G. *Anal. Biochem.* **1973**, *54*, 484–489.
34. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265–275.
35. Craige, J. S.; Wen, Z. C.; Meer, J. P. *Bot. Mar.* **1981**, *27*, 55–61.
36. Jones, T. M.; Albersheim, P. *Plant Physiol.* **1972**, *49*, 926–936.
37. Ciucanu, I.; Kerek, F. *Carbohydr. Res.* **1984**, *131*, 209–217.
38. Sambrook, J.; Fritsch, E. F.; Maniatis, T. In *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory Press: Woodbury, NY, 1982; p 191.
39. Mayer, M. M. Complement and Complement Fixation. In *Experimental Immunochemistry*; Kabat, E. A., Mayer, M. M., Eds., 2nd ed.; Springfield Publications: Kalamazoo, MI, 1961; pp 133–240.
40. Klerx, J. P.; Beukelman, C. J.; Van, D. H.; Willers, J. M. *J. Immunol. Methods* **1983**, *63*, 215–220.
41. Zhou, J.; Zhang, Y. Y.; Zhang, J. W.; Xu, H.; Chen, D. F. *J. Fudan Univ. (Medical Sciences)* **2006**, *33*, 101–106.