Research article

Ultrasound-assisted extraction of polysaccharides from Ginkgo biloba: Process optimization, composition and anti-inflammatory activity

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ABSTRACT

Plant derived polysaccharides can enhance immune function in the human body, effectively prevent diseases, and reduce the probability of bacterial infections. Ginkgo crude polysaccharide (GCP) was obtained from Ginkgo biloba by ultrasonic-assisted hot water extraction. Our data showed that the best extraction conditions of GCP were as follows: extraction temperature 80 °C, ultrasonic time 35 min, extraction time 3 h, and solid–liquid ratio 1:30. Fourier transform infrared spectrometer (FT-IR) data showed that this polysaccharide might be an acidic polysaccharide with a carboxylic acid ring structure. Further studies implied that GCP was mainly composed of glucose, galacturonic acid, rhamnose, galactose and arabinose, accounting for 39.45 %, 25.01 %, 15.40 %, 11.94 % and 4.25 %, respectively. 0.1, 1 and 10 mg/mL GCP reduced the release of inflammatory factors in RAW264.7 cells via inhibition of the nuclear factor kappa-light-chain-enhancer of activated B (NF-κB) signalling pathway. GCP was separated into five components with different molecular weights by an ultrafiltration membrane. Our data showed that GPa with a molecular weight ≥100 kDa was the main component of GCP. 1 mg/mL GPa, GPb, GPc and GPd had anti-inflammatory activities, and 1 mg/mL GPa had the best anti-inflammatory activities. Our results preliminarily reveal the elements and biological activity of GCP, which will provide a reference for the development of Ginkgo biloba.

1. Introduction

Ginkgo is one of the largest medicinal plants in the world and is considered the national tree of China [1]. The medicinal parts of Ginkgo biloba are its leaves and biloba. It contains over 60 bioactive compounds, which improve its medicinal and edible value [2].

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The active compounds in Ginkgo biloba show anti-inflammatory, antioxidant, neuroprotective, antidiabetic, antiatherogenic and cardioprotective effects [3]. At present, ginkgo leaf polysaccharides have been suggested to protect the liver, stimulating immune activity and antioxidation [4,5]. However, there are few studies on the extraction methods and biological activities of polysaccharides in Ginkgo biloba, which need to be elucidated.

Polysaccharides are widely present substances in living organisms, which are a type of natural high molecular weight polymer composed of aldose or ketose linked by glycosidic bonds. They are important biomolecules in living organisms and are one of the basic substances that maintain the normal operation of life activities [6]. Polysaccharides are natural carbohydrate biopolymers that are highly present in animals, plants, fungi, bacteria and seaweeds [7]. Polysaccharides are macromolecules formed by dehydration and condensation of more than 10 monosaccharides through glycosidic bonds [8]. Recently, polysaccharides from plants have been widely studied because of their special biological activities [9]. The extraction methods of polysaccharides mainly focus on the procedure during preparation, which may change the physiochemical activities of polysaccharides and affect their functions [10].

Ultrasound-assisted extraction (UAE) is an effective extraction technology that is applied in the medicine and food industries [11–13]. It can significantly shorten the extraction time, increase yield, reduce energy consumption and save cost. Moreover, the impact on molecular properties and biological activity of polysaccharides obtained by ultrasound-assisted extraction is relatively small [10,12]. Ultrasonic-assisted extraction may also degradation or modify plant polysaccharides with different physicochemical properties and bioactivities by affecting their chemical or spatial structures.

The objective of present work was to extract the active ingredients and explore their activity mechanisms. In this paper, the polysaccharides in Ginkgo biloba were extracted by ultrasonic-assisted extraction, and the process conditions were optimized by orthogonal experiments. The monosaccharide components of GCP were also identified. Further studies showed that GCP reduced the release of inflammatory factors in RAW264.7 cells via inhibition of the nuclear factor kappa-light-chain-enhancer of activated B (NF-κB) signalling pathway. The GCP was separated into five components by an ultrafiltration membrane. GPa, GPb, GPc and GPd had anti-inflammatory activities, and GPa had the best anti-inflammatory activities.

2. Methods and materials

2.1. Reagents and antibodies

LPS (L4391) was purchased from Sigma−Aldrich (Darmstadt, Germany). Cell Counting Kit-8 (ST1007) was obtained from Saint Biology (Shanghai, China). Protein loading buffer (5 × P0015F) was purchased from Beyotime Biotechnology (Shanghai, China). Enzyme-linked immunosorbent assay (ELISA) kits (SEKM-0034, SEKM-0002 and SEKM-0007) were obtained from Solarbio Science & Technology Co., Ltd. (Beijing, China). NF-κB p65 (D14E12), phospho-NF-κB p65 (Ser536) (93H1), NF-κB inhibitor alpha (IκBα) (L35A5) and phospho-IκBα (Ser32) (14D4) were purchased from Cell Signaling Technology (Danvers, United States). Goat anti-mouse IgG (1070-05) and goat anti-rabbit (4050-05) antibodies were obtained from SouthernBiotech (Birmingham, United States).

2.2. Cell culture

The RAW264.7 cell line was purchased from the Cell Bank of the Chinese Academy of Sciences and incubated in Roswell Park Memorial Institute (RPMI) 1640 with 10 % foetal bovine serum (FBS) (BI, Israel) at 37 °C and 5 % CO_2. RAW264.7 cells exhibit two cell morphologies: monocytes (spindle shaped) and macrophages (round shaped). The higher the density of cell growth, the more macrophages (round cells) there will be. So it is best to use high-density cell culture and perform cell passage at a density of 90 %–100 %. 1–3 mL of complete culture medium was used to blow and wash off the adherent cells (most round cells can be washed off) when passaging. If most of the cells fell off, the cells were gently blown and evenly distributed. The cell suspension was transferred to two new culture bottles and added 8–10 mL new complete culture medium. The detached cells were collected by centrifugation at 1000 rpm for 5 min and mixed with the dispersed cells for subculture.

2.3. Extraction of crude polysaccharide from ginkgo biloba

Ginkgo biloba powder was purchased from Handan Zhenhuitang Pharmaceutical Co., Ltd. (Hebei, China) and placed into a glass tube for ultrasonication after the addition of ultrapure water. GCP was extracted through one-factor experiments under these conditions: extraction temperatures were 60, 70, 80, 90 and 100 °C; ultrasonic times were 5, 15, 25, 35 and 45 min; extraction times were 1, 2, 3, 4 and 5 h; and solid−liquid ratios were 1:10, 1:20, 1:30, 1:40 and 1:50. The crude samples were extracted in a water bath after being ultrasonicated for the indicated times. These samples were centrifuged at 5000 rpm for 5 min, and the supernatants were collected. The proteins in these samples were removed with trichloroacetic acid (TCA). Then, the crude ginkgo polysaccharides were obtained and stored in −80 °C after being precipitated in 80 % alcohol overnight. The extraction rate formula of GCP was as follows:

Extraction yield (%) = m/M* 100%

"m" indicates the quality of GCP, and M is the quality of ginkgo powder.

2.4. Optimization of extraction conditions by orthogonal test

Single-factor experiments, including extraction temperature (A), ultrasonic time (B), extraction time (C), and solid−liquid ratio (D), were conducted to determine the preliminary range of extraction factors. The optimal extraction conditions were determined by orthogonal experiments. The extraction conditions for GCP are shown in Table 1.

2.5. Fourier transform infrared spectroscopy (FT-IR) analysis

The ginkgo crude polysaccharide was mixed with potassium bromide powder in a mass ratio of 1:100 and placed in an air-drying oven for 6 h. Then, the mixtures were crushed in a mortar for testing by FT-IR.

2.6. Analysis of the monosaccharide composition of GCP

A total of 5 0.00 ± 0.05 mg polysaccharide was placed in a clean chromatography bottle. The sample was heated at 121 °C for 2 h after the addition of 1 mL of 2 M TFA acid solution. Then, the sample was purified with methanol 3 times after being dried with nitrogen. Finally, the sample was transferred to a chromatography vial for the test.

2.7. Instruments

Dionex ™ CarboPac ™ PA20 liquid chromatographic column. Thermo ics5000 ion chromatographic system. FTIR (PerkinElmer, Norwalk, CT, USA). Multiskan SkyHigh full wavelength ELISA reader (Thermo Fisher Scientific Inc., A51119500C). Luminescent Image Analyzer (GE Healthcare Bio-Sciences, Uppsala, Sweden).

2.8. Ultrafiltration classification

The GCP was loaded into the feed liquid tank using mini pelican ultrafiltration equipment. Then, the intercepted liquid was ultrafiltered after reaching the ultrafiltration membrane. The GCP was separated and concentrated after the flow rate and the reflux valve were adjusted to control the operating pressure. Each ultrafiltration membrane (with specifications of 100, 50, 30 or 10 kDa) was used to ultrafiltrate the extraction at room temperature. Then, the extraction solution was evaporated, prefrozen at −80 °C, and freeze-dried. The crude ginkgo polysaccharide was divided into five fractions: GPa ≥100 kDa, 100 kDa ≥ GPb ≥50 kDa, 50 kDa ≥ GPc ≥30 kDa, 30 kDa ≥ GPd ≥10 kDa, and GPe ≤10 kDa.

2.9. Test of total sugar content

The total sugar was tested by the anthrone-sulfuric acid method. Briefly, 0, 0.2, 0.4, 0.6, 0.8, and 1 mg/mL glucose standard solutions and 1 mg/mL sample solutions were prepared. Then, 6 mL of anthrone sulfuric acid was added to each solution, and the absorbance value was immediately measured at 625 nm.

2.10. Cell viability assay

RAW264.7 cells were incubated in 96-well plates at 37 °C, 5 % CO_2 for 24 h and washed with phosphate-buffered saline (PBS). The cells were washed with phosphate-buffered saline (PBS). Then, PBS was discarded, 100 μL serum-free medium containing cell counting kit-8 (CCK-8) reagent was added. The culture plates were incubated in the incubator at 37 °C for 1–4 h. And the optical density (OD) value of cell plate was measured at 450 nm using an enzyme marker.

2.11. Measurement of the release of inflammatory factors

RAW264.7 cells were incubated in 48-well plates, and the supernatant was collected in another plate. Standard wells and sample wells were setted. 50 μL of standard samples with different concentrations were added to each standard well. The wells without sample or enzyme-linked immunosorbent assay were taken as blank control and test sample wells respectively. 40 μL sample diluent was added to the well of the test sample. 10 μL samples (with a final dilution of 5 times) were added to the bottom of the enzyme-linked immunosorbent assay plate wells and gently shaken and mixed well, avoiding touching the well wall as much as possible. 100 μL

Table 1
Orthogonal test.

| Level	Factor			
	Extraction temperature (°C)	Ultrasonic time (min)	Extraction time (h)	solid−liquid ratio (g/mL)
1	70	25	1	1:30
2	80	35	2	1:40
3	90	45	3	1:50

enzyme labeled reagents recognized TNF-α, IL-1β or IL-6 were added to each well, except for blank wells. The plates were sealed with sealing film and incubate at 37 °C for 60 min. The samples were diluted 20 times with distilled water. Then, the sealing film was removed and the liquid was discarded. Each hole was filled with detergent and incubated for 30 s. This process was repeated 5 times. 50 μL color reagent was added to each well and incubated at 37 °C in the dark for 15 min 50 μL termination solution was added to each well to terminate the reaction. The absorbance value of each hole was measured at a wavelength of 450 nm. The measurement should be conducted within 15 min after the termination solution being added.

2.12. Test the total polyphenol content

The total polyphenol content in plant extracts and their products was determined using the method of folin phenol spectrophotometric. Phenolic compounds reduce phosphotungstic molybdic acid under alkaline conditions to form blue compounds. Within a certain concentration range, the absorbance value is directly proportional to the content of phenolic compounds, in accordance with Lambert Beer's law. The concentration of total polyphenols in the test solution was calculated based on the standard curve by measuring the absorbance value of the sample.

2.13. Western blotting

RAW264.7 cells were incubated in 6-well plates. The cells were washed with PBS 3 times and lysed in 200 μL of 2 % SDS. Then, the extracts were boiled at 95 °C for 10–25 min, and 50 μL of 5 × protein loading buffer was added. The extracts were heated at 95 °C for 10–25 min. The proteins were transferred to a polyvinylidene difluoride (PVDF) membrane after being separated by SDS−PAGE. The membrane was incubated with primary antibodies and secondary antibodies. Finally, the membrane was placed in a small box with electrochemiluminescence (ECL) and imaged using a Luminescent Image Analyser.
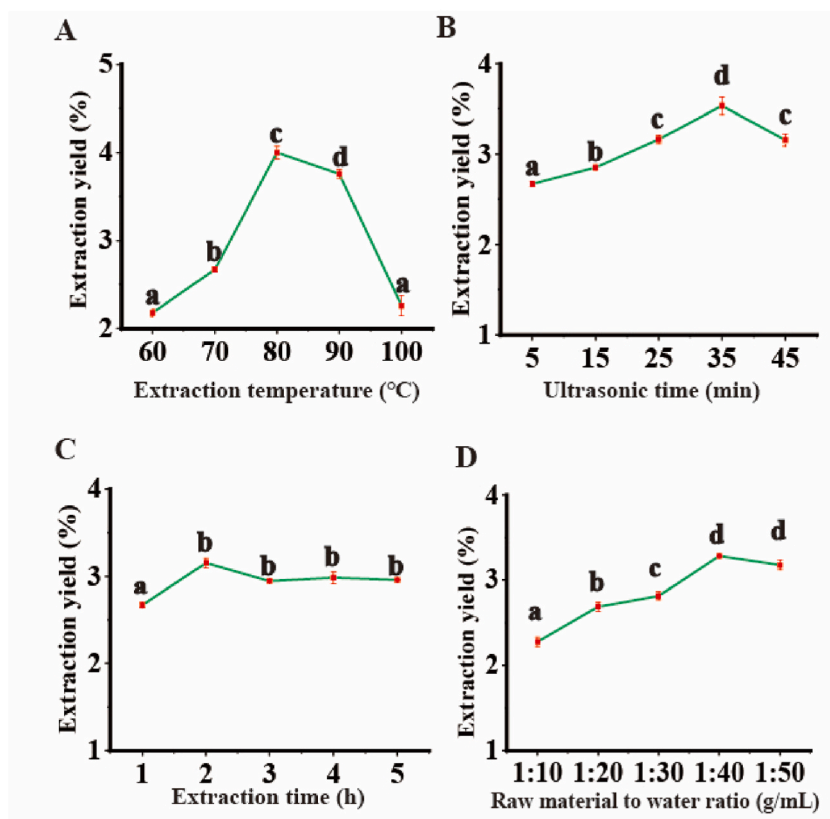
Fig. 1. Optimization of extraction processes of polysaccharides from Ginkgo biloba (A) The impact of extraction temperature on the extraction rate of polysaccharides from Ginkgo biloba. (B) The effect of ultrasonic waves on the extraction rate of polysaccharides from Ginkgo biloba. (C) The impact of extraction time on the extraction rate of polysaccharides from Ginkgo biloba. (D) The effect of solid−liquid ratio on the extraction rate of polysaccharides. Different letters in a curve represent significant differences (P < 0.05). Each experimental group was tested at least 3 times.

2.14. Statistical analysis

Data was analyzed by the software of SPSS11.0 Software. Each experiment were performed 3 times. Each group has at least 3 repeats. Data was expressed as the mean $\pm$ S.D. One-way ANOVA was used to measure statistical differences between the means within each experiment. Different letters mean differences are distinctive ($p < 0.05$) compared with control group.

3. Results and disussion

3.1. Impact of single-factor experiments on the extraction rate of ginkgo polysaccharides

The best parameters of these four planes, including extraction temperature, ultrasonic time, extraction time and solid−liquid ratio, were taken as the central point of the orthogonal experiment (Fig. 1A–D). The extraction rate showed a significant increase when the extraction temperature was in the range of 60 °C–80 °C. The extraction rate was highest when the extraction temperature reached 80 °C (4.00 % $\pm$ 0.07 %), and then decreased with increasing temperature. The reason might be that extraction at high temperature accelerates the outflow rate of polysaccharides from cells, the movement rate between molecules increases, and more polysaccharides can dissolve into the solution. However, if the extraction temperature is too high, other substances that are slightly soluble in the solution system will also dissolve in the system, and will be precipitated when dissolved with water. Moreover, extraction at high temperature may also damage the structure of polysaccharides, leading to their degradation and a decrease in the extraction rate (Fig. 2A). As shown in Fig. 1B, the extraction rate of polysaccharides showed a significant upward trend with the prolongation of ultrasound time and reached the maximum value (3.53 % $\pm$ 0.10 %) at ultrasound time of 35 min. It can be speculated that the longer the ultrasound time, the greater the degree of damage to the cell structure, which allows for better release of intracellular substances and increases the extraction rate. However, it was found that the extraction rate of polysaccharides from Ginkgo biloba actually decreased by further extending the ultrasound time, which may be due to the degradation of polysaccharides caused by excessive ultrasound time (Fig. 2B). The extraction rate of polysaccharides reached its maximum value (3.15 % $\pm$ 0.06 %) when the extraction time was 2 h, which meant that the polysaccharides were fully dissolved. The extraction rate slightly decreased, but there was no significant difference with the extension of extraction time. The reason might be that the thermal instability of polysaccharides caused
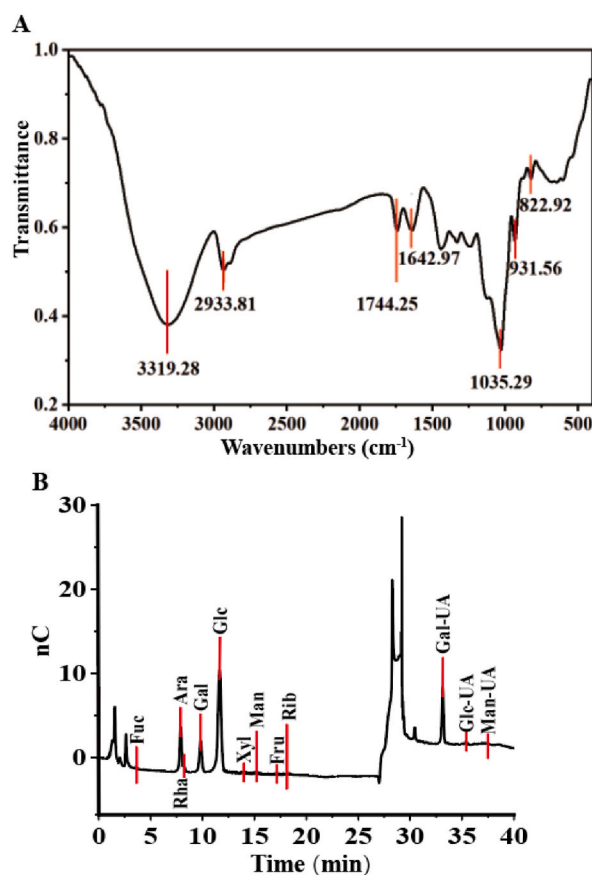
Fig. 2. Composition and group analysis of polysaccharides. (A) Fourier transform infrared spectrum of polysaccharides from Ginkgo biloba. (B) Analysis of the monosaccharide composition of GCP by ion chromatography.

by long-term extraction, which leads to its degradation. Therefore, when the extraction time is 2 h, polysaccharides may have been completely extracted. The higher the material to water ratio, the higher the extraction rate of polysaccharides (Fig. 2C). The extraction rate of polysaccharides reached its maximum value (3.283 % ± 0.006 %) when the material to water ratio was 1:40. It implies that increasing the material to water ratio can reduce the viscosity of the solution, improve the diffusion rate of internal and external solvents in the raw materials, increase the solubility of polysaccharides and ultimately enhance the extraction rate of polysaccharides (Fig. 2D).

Our data showed that the relationship between the four factors on the range R is: temperature > ultrasonic time > solid–liquid ratio > extraction time. The factors that affect the extraction rate of crude polysaccharides from white fruit are in the following order: extraction temperature > ultrasonic time > material water ratio > extraction time. The optimal extraction conditions for GCP were an extraction temperature of 80 °C, ultrasonic time of 35 min, extraction time of 3 h, and solid–liquid ratio of 1:30 (Table 2). Meanwhile, we conducted multiple linear regression equation analysis on the above data (Table 3). Although Hu et al. verified that the thermal effects during ultrasonic irradiation would not affect polysaccharide change if the internal temperature of ultrasonication was not over 100 °C [14]. Here we reported that temperature has an impact on the extraction rate. The higher the temperature, the higher the extraction rate when the extraction temperature is below 80 °C. The higher the temperature, the lower the extraction rate when the extraction temperature is above 80 °C.

3.2. Identification of polysaccharide groups and monosaccharide composition

To investigate the structure and monosaccharide composition of GCP, we used Fourier transform infrared spectroscopy (FT-IR) and ion chromatography to test the groups and monosaccharide composition of GCP. The FT-IR spectrum of GCP shown in Fig. 2A revealed a strong absorption band at 3319.28 cm^{-1}, corresponding to -OH. The absorption peak at 2933.81 cm^{-1} corresponded to the -CH stretching vibration. The peaks at 1744.25 cm^{-1} and 1642.97 cm^{-1} were carbonyl and C=O, which indicated that the GCP contained carbonyl and -COOR groups. Combined with 3319.28 cm^{-1} and 931.56 cm^{-1}, it can be concluded that the polysaccharide had a carboxylic acid group (-COOH). The peak at 1035.29 cm^{-1} was the hydroxyl-carbon oxygen (C-O) of alcohol, which was also a characteristic absorption peak containing hydroxyl groups in a molecule. Sugars with ring structures had absorption components at 900-700 cm^{-1}. The peak at 822.92 cm^{-1} indicated that the crude ginkgo polysaccharide could be a polysaccharide with a ring structure.

The ion chromatographic spectrum of GCP shown in Fig. 2B revealed that the crude polysaccharide of Ginkgo biloba was mainly composed of glucose, galacturonic acid, rhamnose, galactose and arabinose.

To further analyze the content of monosaccharides, we prepared standard solutions of different concentrations (Table 4). The concentration of the standard was plotted on the x-axis, and the peak area of the standard was plotted on the y-axis. The mathematical relationship between the target compound and its peak area was obtained. Then, each concentration of monosaccharides based on the peak area of the corresponding compound in the unknown sample were calculated. Our data showed that the five monosaccharides account for 39.45 %, 25.01 %, 15.40 %, 11.94 % and 4.25 %, respectively (Table 5 and Fig. 2B).

3.3. GCP inhibited cellular inflammation

To ensure that the concentrations of LPS and GCP did not affect cell viability, we used the CCK-8 method to test the effect of LPS and GCP on the viability of RAW264.7 cells. As shown in Fig. 3A, the viability rate was not changed in the 0.1, 1 or 10 μg/mL LPS-treated groups compared with the control group. The rate of viability was decreased in the 20 μg/mL LPS-treated group. GCP (0.1, 1 and 10 mg/mL) did not affect the viability of RAW264.7 cells (Fig. 3 B).

Table 2
Optimization of crude polysaccharide extraction from white biloba with results of orthogonal tests.

Test number	Factor				
	Extraction temperature (°C)	Ultrasonic time (min)	Extraction time (h)	solid-liquid ratio (g/mL)	extraction rate(%)
1	70	25	1	1:30	3.24
2	70	35	2	1:40	3.53
3	70	45	3	1:50	3.82
4	80	25	2	1:50	4.09
5	80	35	3	1:30	4.50
6	80	45	1	1:40	3.81
7	90	25	3	1:40	2.82
8	90	35	1	1:50	3.60
9	90	45	2	1:30	2.62
K1	10.60	10.15	10.65	10.37	–
K2	12.41	11.64	10.24	10.16	–
K3	9.05	10.25	11.15	11.51	–
k1	3.53	3.88	3.55	3.45	
k2	4.14	3.88	3.41	3.39	
k3	3.02	3.42	3.72	3.84	
R	1.12	0.50	0.30	0.45	

Table 3

Analysis of multiple linear regression.

ANOVA					
Reource of variance	Sum of Squares	Degree of Freedom	Mean Aquare	F Value	Significance
Interception	342.26	1	342.26	29287.23	0
Extraction temperature	6.50	2	3.25	277.93	0
Ultrasonic time	1.01	2	0.51	43.40	0
Extraction time	0.27	2	0.14	11.62	0.001
solid-liquid ratio	1.08	2	0.54	46.07	0
Error	0.21	18	0.12		
Total	9.07	26			

Table 4

Preparation of standard samples with different concentrations of monosaccharides.

Name	Concentration (µg/mL)							
Fucose	0.4	0.8	4	8	16	24	32	40
Rhamnose	0.4	0.8	4	8	16	24	32	40
Arabinose	0.4	0.8	4	8	16	24	32	40
Galactose	0.4	0.8	4	8	16	24	32	40
Glucose	0.4	0.8	4	8	16	24	32	40
Xylose	0.4	0.8	4	8	16	24	32	40
Mannose	0.5	1	5	10	20	30	40	50
Fructose	0.6	1.2	6	12	24	36	48	60
Ribose	0.6	1.2	6	12	24	36	48	60
Galacturonic Acid	0.6	1.2	6	12	24	36	48	60
Glucuronic Acid	0.6	1.2	6	12	24	36	48	60
Glucuronic Acid	0.6	1.2	6	12	24	36	48	60
Mannuronic Acid	0.6	1.2	6	12	24	36	48	60

To evaluate the anti-inflammatory effect of GCP, we used ELISA to detect the secretion of proinflammatory factors after the RAW264.7 cell lines were treated with LPS plus the indicated concentration of GCP. The results showed that GCP decreased the release of TNF-α, IL-6 and IL-1β (Fig. 3C and D and E).

3.4. GCP inhibited the release of inflammatory factors via the NF-κB signalling pathway

p65 is phosphorylated and transported into the nucleus. IκBα is also phosphorylated and then degraded by the proteasome once NF-κB signalling is activated [15]. As shown in Fig. 4A and B, LPS increased the phosphorylation of p65 and IκBα. The phosphorylation of p65 and IκBα was decreased in the LPS plus GCP-treated group.

3.5. GPa showed a better anti-inflammatory effect

Polysaccharides are a mixture consisting of several substances with different degrees of polymerization. Polysaccharides with different molecular weights have different biological activities [16]. The crude polysaccharide of Ginkgo biloba was separated into various components with different molecular weights by an ultrafiltration membrane: GPa ≥100 kDa, 100 kDa ≥ GPb ≥50 kDa, 50 kDa ≥ GPc ≥30 kDa, 30 kDa ≥ GPd ≥10 kDa, and GPe ≤10 kDa.

Our data showed that GPa with a molecular weight ≥100 kDa was the main component of Ginkgo crude polysaccharide (Table 6). To explore the anti-inflammatory effects of the 5 polysaccharides, the cell viability and release of inflammatory factors were tested after the cells were treated with LPS plus the 5 polysaccharides. The results showed that the five GPs at a concentration of 1 mg/mL did not affect cell viability, indicating that the five GPs at this concentration do not have cytotoxicity (Fig. 5A). LPS increased the release of TNF-α, IL-6 and IL-1β. The release of TNF-α, IL-6 or IL-1β was decreased after the cells were treated with GPa, GPb, GPc or GPd (Fig. 5B, C and D). These data show that GPa, GPb, GPc and GPd have anti-inflammatory activities, and GPa shows the best anti-inflammatory activities.

Inflammation is a protective response to stimuli [17]. The clinical manifestations of inflammation are redness, swelling, heat, pain, and dysfunction. Inflammatory reactions involve many cell signalling pathways and immune cells. The inflammatory process occurs in living tissues with a vascular system, primarily in response to various injury factors, resulting in defence responses, including enhanced blood vessel permeability and leukocytes percolating into the injured tissues guided by cytokines [18]. Inflammation can be classified into two classes: acute and chronic inflammation. Chronic inflammation may be initiated by ongoing, long-term host exposure to stimuli [19]. The persistent presence of proinflammatory factors and tissue damage or acute inflammation not receiving timely and effective treatment are the fundamental causes of chronic inflammation [19]. Acute inflammation is a short process with three steps; first there is enhanced blood flow to the injured area, followed by vasodilatation and enhanced vascular permeability, and inflammatory cells such as lymphocyte cells and mononuclear macrophages migrate to the injured tissue along with other inflammatory

Table 5
Composition and content of monosaccharide in GCP.

| monosaccharide	fucose	rhamnose	arabinose	galactose	glucose	xylose	mannose	fructose	ribose	Galactose uronic acid	Glucuronic acid	mannuronic acid
Content (μg/mg)	0.58	17.99	4.97	13.95	46.09	0.39	0.93	0.51	0.84	29.23	0.48	0.87
Percentag (%)	0.50	15.40	4.25	11.94	39.45	0.33	0.80	0.44	0.72	25.01	0.41	0.74
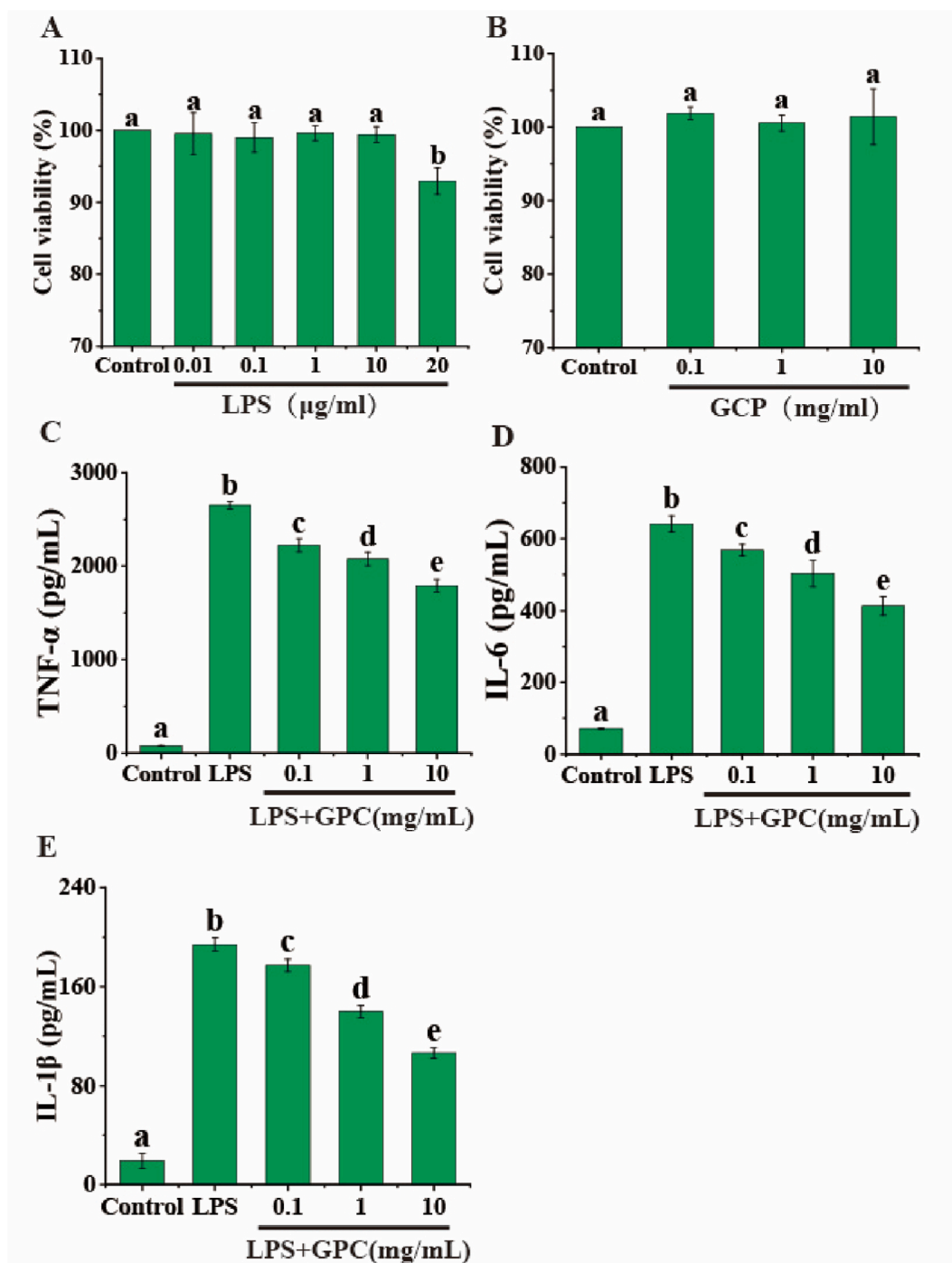
Fig. 3. GCP inhibited the release of inflammatory factors in RAW264 7 cells. (A) The RAW264.7 cells were treated with 0.1, 1 or 10 μg/mL LPS for 12 h, and the cell viability rate was analyzed by the method of CCK-8. (B) The RAW264.7 cells were treated with 0.1, 1 and 10 mg/mL with GCP for 12 h, and the cell viability was analyzed by the CCK-8. (C)–(E) The RAW264.7 cells were treated with or without 10 μg/mL LPS, 10 μg/mL LPS plus 0.1 mg/mL, 1 mg/mL or 10 mg/mL GCP for 12 h and the concentration of TNF-α (C), IL -6 (D) or IL -1β (E) was determined by the method ELISA. Different letters mean differences are distinctive (p < 0.05). Each experimental group was done at least 3 times.

factors [18].

Inflammation is a complex process that is regulated by cytokines. The first process of the inflammatory mechanism is the recognition of infections by transmembrane proteins such as Toll-like receptors (TLRs) and intracellular nucleotide-binding domain and leucine-rich-repeat-containing receptors (NOD-like receptors or NLRs) [20]. The signal is transduced to the cell, and NF-κB is
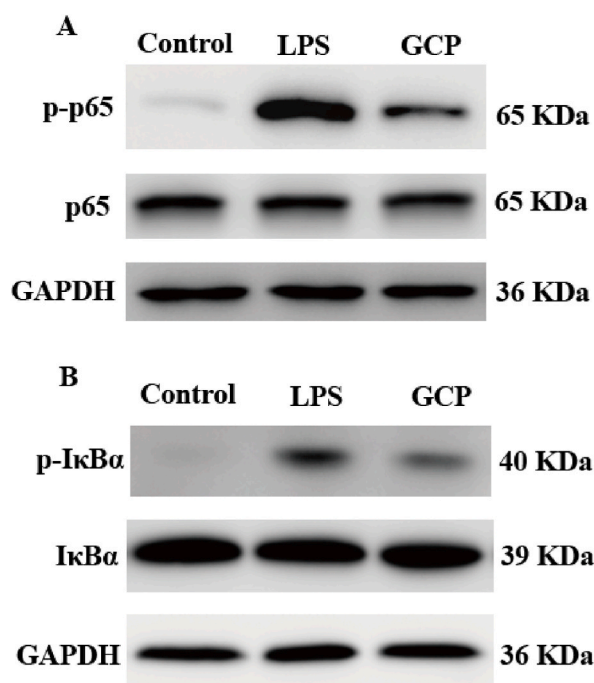
Fig. 4. GCP inhibited the release of inflammatory factors in RAW264.7 cells via the NF-κB signalling pathway. (A) RAW264.7 cells were treated with or without 10 μg/mL LPS or 10 μg/mL LPS plus 10 mg/mL GCP for 12 h, and the expression of p65 or phospho-p65 was immunoblotted with antibodies against phospho-p65 and p65. (B) RAW264.7 cells were treated with or without 10 μg/mL LPS or 10 μg/mL LPS plus 10 mg/mL GCP for 12 h, and the expression of IκBα or phospho-IκBα was immunoblotted with antibodies against phospho-IκBα and IκBα. GAPDH was used as negative control.

Table 6

Quality of Ginkgo polysaccharide before and after ultrafiltration.

Ginkgo polysaccharide components	quality(g)
Total weight	2.336
GPa	1.073
GPb	0.072
GPc	0.073
GPd	0.025
GPe	0.074

activated. Then, this transcription factor is released from IκB and enters the nucleus to induce the expression of cytokines. These cytokines can promote inflammation and recruit immunocytes such as macrophages, mast cells and neutrophils to damaged areas [21]. Macrophages can engulf pathogenic bacteria, digestive bacteria and impaired host cells [22]. Neutrophils release chemicals to block microbial invasion and destroy host cells [23]. The final process of inflammation is its termination to reduce the damage to host cells. Inflammation is a normal response in the body, but excessive or prolonged inflammatory reactions pose many health hazards. Severe hypersensitivity inflammation can threaten the patient's life [24]. In addition, the inflammation of special parts or organs can cause serious consequences, such as damage to the intestines, lungs and joints, even leading to the occurrence of cancer [25–28]. It has been reported that polysaccharide can inhibit the inflammatory process via Keap1-Nrf2/HO-1, FoxO3a/Autophagy, RIPK1-mediated NF-κB, FoxO3a/Autophagy, TLR4/NF-κB and ERK activation [29–32]. We reported that GCP inhibited the release of inflammatory factors via the NF-κB signalling pathway and the molecular mechanism needs to be explored.

Polysaccharides are composed of ten or more different monosaccharides. They can be obtained from multiple organisms, including marine organisms, plants, bacteria, fungi, animals and even humans, and have become a research hotspot[10]. Polysaccharides have several biological activities [33]. Therefore, many polysaccharides are widely available in the food and pharmaceutical fields because of their good bioactivity [34]. The physical activities of polysaccharides depend on their structural features [35]. In this paper, the average molecular weight of polysaccharide was determined by high performance gel permeation chromatography (HPGPC). The results indicated that its molecular weight was 831.95 Da. The total polyphenol content in this extract was tested by the method of folin phenol spectrophotometric. The results showed that the total polyphenol content in the polysaccharide is 0.75 % and it contained no flavone. We also confirmed that the crude polysaccharide of Ginkgo biloba was mainly composed of glucose, galacturonic acid, rhamnose, galactose and arabinose. The five monosaccharides account for 39.45 %, 25.01 %, 15.40 %, 11.94 % and 4.25 %,
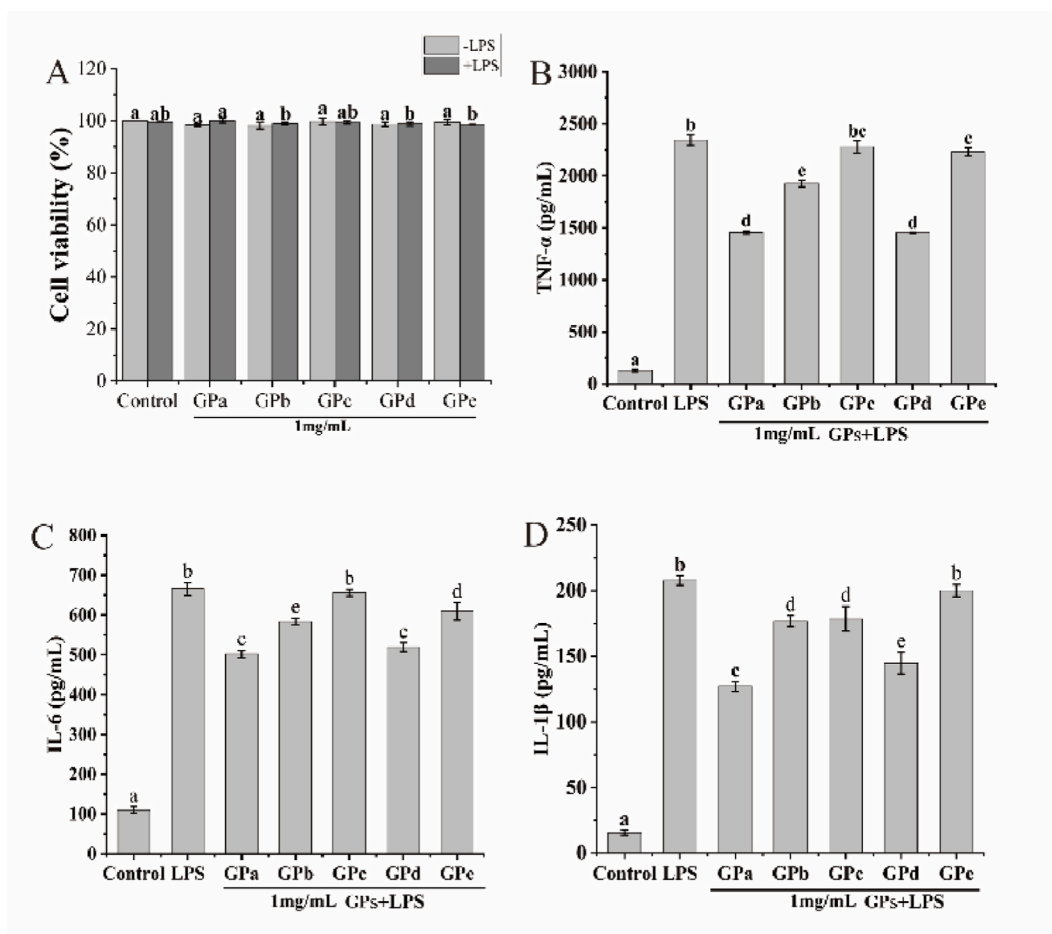
Fig. 5. The effect of GPs on the LPS-induced release of inflammatory factors. (A) RAW264.7 cells were treated with or without 1 mg/mL GPs, and the rate of cell viability was tested by CCK-8. RAW264.7 cells were treated with or without 1 mg/mL GPs plus 10 μg/mL LPS for 12 h, and the release of inflammatory factors, including TNF-α (B), IL-6 (C) and IL-1β (D), was determined by ELISA. Different letters indicate significant differences (P < 0.05). Each experimental group was tested at least 3 times.

respectively. Then, this crude polysaccharide of Ginkgo biloba was separated into different components with different molecular weights by an ultrafiltration membrane. GPa, GPb, GPc and GPd had anti-inflammatory activities, and GPa showed the best anti-inflammatory activities. It is well known that molecular weight, chain conformation, the composition of monosaccharides and functional groups in the structure are essential factors that significantly affect the functions of polysaccharides. The structural features such as monosaccharide, molecular weight, chemical bonds and functional groups also have effect on anti-inflammatory activity of Ginkgo biloba polysaccharide [14]. Phenolic groups in polysaccharide of Ginkgo biloba may play a certain role in anti-inflammatory activity. However, the structure and chain conformation of the polysaccharide from Ginkgo biloba remain to be explored.

There are several methods for polysaccharide extraction, such as enzyme extraction, ultrasonic-assisted extraction, microwave assisted extraction, high-pressure pulse extraction, supercritical fluid extraction, etc. Each method has its own characteristics and applicability. The selection of an appropriate extraction method depends on factors such as the properties of the polysaccharide, the purity requirements of the target product, and production costs [35]. An appropriate extraction method increases the extraction yield and influences the biological activity of polysaccharides [36,37]. In this paper, the polysaccharides in Ginkgo biloba were extracted by ultrasonic-assisted extraction, and the optimal conditions were obtained by orthogonal experiments. The polysaccharide from Ginkgo biloba showed good anti-inflammatory activity. Ultrasound can disrupt and destroy the cell wall of the plant matrix and improve the contact between the solvent and the target compound to promote the release of bioactive compounds to accelerate the extraction rate [38]. It has been reported that ultrasonic power, long ultrasonic time, high ultrasonic frequency, temperature and intensity have effects on plant polysaccharides [14]. Apart from physical methods, microbial fermentation could also improve the extraction efficiency of Ginkgo biloba polysaccharide [39]. So, which extraction method is the best for extracting polysaccharides from Ginkgo biloba remains to be clarified. The polysaccharides in Ginkgo biloba possess non-toxicity and easily obtainable properties, which will make them extensively used in release drug delivery systems, improving the quality of yoghurt and as a disintegrating agent.

4. Conclusions

Our data showed that an extraction temperature of 80 °C, ultrasonication time of 35 min, extraction time of 3 h and solid−liquid ratio of 1:30 were the optimal extraction conditions for GCP. The crude polysaccharide of Ginkgo biloba was mainly composed of glucose, galacturonic acid, rhamnose, galactose and arabinose. Further studies showed that GCP reduced the release of inflammatory factors via the NF-κB signalling pathway. GCP was also separated into five components with different molecular weights by an ultrafiltration membrane. Our data showed that GPa with a molecular weight ≥100 kDa was the main component of GCP. GPa, GPb, GPc and GPd all had anti-inflammatory activities, and GPa had the best anti-inflammatory activity. Our results preliminarily reveal the elements and biological activity of GCP, which will provide a reference for the development of Ginkgo biloba.

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Data availability statement

The data generated in our study are not deposited into a publicly available repository, which are available from the first author on reasonable request.

CRediT authorship contribution statement

Mengzhi Zhang: Writing – original draft, Methodology. **Yifei Wang:** Methodology, Conceptualization. **Qiuyi Li:** Methodology, Formal analysis, Data curation. **Yunfang Luo:** Formal analysis, Data curation. **Li Tao:** Software, Resources. **Dengli Lai:** Validation, Software, Resources. **Yu Zhang:** Software, Methodology. **Ling Chu:** Validation, Software, Data curation. **Qingwu Shen:** Project administration, Investigation. **Dongbo Liu:** Project administration, Investigation, Data curation. **Yanyang Wu:** Writing – review & editing, Supervision, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Yanyang Wu reports financial support was provided by National Natural Science Foundation of China. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

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