Isolation and identification of anti-inflammatory and analgesic polysaccharides from Coix seed *(Coix lacryma-jobi L.var. Ma-yuen (Roman.) Stapf)*

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Abstract: Coix seed is a nutrient-rich food and traditional Chinese medicine with anti-inflammatory and analgesic properties. Polysaccharides from Coix seed have been rarely investigated for structure and activities. In this study, the analgesic and anti-inflammatory effects were investigated in vivo and in vitro. The results showed that Coix seed had a significant influence on reducing the number of writhing, increasing the pain threshold and alleviating the swelling degree caused by acute inflammation. Column chromatography was used to obtain two active compounds of Coix seed. Compound 1 was (1→6)-α-D-glucan with a molecular weight of 6.81×10^5 Da. The chemical connection of compound 2 was as follows: α-Frup (2→ [1)-α-Glcp (6]$_5$→1)-α-Glcp (4→1)-α-Glcp, which was isolated in Coix seed for the first time. LPS-induced inflammation in RAW264.7 cells was well inhibited by compounds. These findings offered a preliminary investigation into the analgesic and anti-inflammatory properties of Coix seed, which may be helpful for application.

Keywords: **Coix seed polysaccharide; analgesic; anti-inflammatory; Structure**

Experimental

Materials and reagents

Coix seed (Coix lacryma-jobi L.var. Ma-yuen (Roman.) Stapf) was purchased from Bozhou, Anhui Province, China. The plant materials were identified by Zenglai Xu at Institute of Botany, Jiangsu Province and Chinese Academy of Sciences (Nanjing, China). The voucher specimen (No. Xudeping20210119-75) was deposited in the Food Quality Research Laboratory, School of Food Science and Technology, Jiangnan University. Sigma-Aldrich Chemical Co., LTD supplied the glacial acetic acid and carrageenan (Shanghai, China), other chemical reagents were purchased from Sinopharming Chemical Reagents (Shanghai, China) Co., LTD. Aspirin enteric-coated tablets were obtained from Bayer HealthCare Manufacturing S.r.l. (Beijing, China). MCI, DEAE-52 and Sephacryl S-400 were ordered from TOSOH Bioscience Shanghai Co., Ltd. (Shanghai, China). Unless otherwise noted, all chemicals employed in this study were of analytical quality.

Extraction and separation of compositions from the Coix seed

The Coix seed powder (10 kg) was extracted with 100 L of distilled water for 3 hours at 60°C. After standing for a while, the extract was filtered, and the ethanol was evaporated at 50°C under decreased pressure. The obtained concentrate was freeze-dried to produce the ethanol extract of Coix seed (CSE), which was stored in the refrigerator at -20°C for subsequent experiments. The filter residue was extracted with distilled water according to the same extraction process(Du et al. 2018). After two times of extraction, the supernatants were mixed and concentrated at 60°C. The aqueous extract was precipitated with three-fold 95% ethanol for 24 hours at 4°C. As a result of centrifugation, the precipitate was washed and lyophilized, yielding crude polysaccharide (CSP-A). Subsequently, the supernatant was then mixed with four volumes of 95% ethanol and precipitated at 4°C overnight. After washing three times of the resulting precipitate with anhydrous ethanol, the precipitation was then lyophilized to collect a polysaccharide with a smaller molecular weight, which was named CSP-B.

Evaluation of analgesic and anti-inflammatory activity

Animals and treatment

Male BABL/c mice weighing 18-22 g were purchased from Gempharmatech Co., Ltd. (Nanjing, China). Five mice in each cage were freely given food and water under standard conditions at the Jiangnan University Experimental Animal Center. The Experimental Animal Ethics Committee of Jiangnan University approved the conditions and operation for the animal experiments (JN. No20211215b0800318[526]). Animals were fed adaptively for seven days before the experiments. The male mice were separated into six groups of ten individuals each at random. The specific groups were as follows: (NC) negative control group, (AC) aspirin positive control group (150 mg/kg, i.g.) (Tadiwos et al. 2017), (MC) model group, (CSE) the ethanol extract group (250mg/kg, i.g.), CSP-A group (250mg/kg, i.g.) and CSP-B group (250mg/kg, i.g.). All samples were dissolved in distilled water. The administration dose was determined from previous studies on Coix seed polysaccharides in other animal studies (Xia et al. 2021). The animals of the negative control group and model group were administrated with distilled water (10 mL/kg, i.g.). All the mice were given the treatment once a day for seven days.

Acetic acid-induced writhing test

The acetic acid-induced writhing test was performed with minor changes to the method of Tadiwosa et al. (Tadiwos et al. 2017). Except for the model group, each animal was injected intraperitoneally with an aqueous solution of 0.6% glacial acetic acid (0.01 mL/g) after one hour of the last dose on the seventh day, and then placed in a big glass container for observation. Following the injection of mice with acetic acid solution, the total number of writhing movements within 15 minutes was counted as an indicator of nociceptive behaviour. To quantify analgesic activity, the percent inhibition of observed writhing movements between the model and treated groups was employed. The calculation formula of percent inhibition was as follows.

% Inhibition = (number of writhing in the model group - number of writhing inthe treated group)/number of writhing in the model group× 100

Hot plate test

The hot plate experiment established by Hu et al. (Hu, Wang, et al. 2022) was conducted to assess thermally-induced discomfort in mice. All mice were pre-tested at 55 ± 0.5℃ on a hot plate. The latency time was defined as the period between licking the paws or jumping reaction. The mice used in the investigation had pain thresholds between 5 and 30 seconds. In order to minimize individual differences among animals and prevent damage to tissues, mice with latency times less than 5 s and greater than 30 s on pretesting were excluded from the subsequent test. Latency times were measured in seconds after mice were placed on a hot plate after 30 min, 60 min, and 90 min of the last treatment.

Xylene-induced ear swelling in mice

The xylene-induced ear swelling model was adapted from the method of Thomas et al. (Thomas et al. 2021) and changed a little. All mice were given the treatment specified in part 2.3.1 for seven days in a row. Except for the negative control group, xylene was equally applied to the inner and outer surfaces of the right ear of mice in all groups one hour after the last administration on the seventh day. All mice were euthanized after 30 minutes of xylene exposure, and both ears were s clipped along the auricles. A hole punch with a diameter of 8 mm was used to punch circular pieces in the same part of the left and right ears, respectively, and then the two ear pieces were weighed. The difference in weight between the two parts of the ear was used to figure out the degree of swelling, and the following formula was employed to compute the swelling inhibition rate:

% Inhibition = (Average swelling degree of the model group -Average swelling degree of the treated group)/Average swelling degree of the model group × 100

Carrageenan-induced paw edema in mice

The method of carrageenan-induced paw inflammation was modified somewhat from that of Liu et al. (Liu et al. 2020). The mice were pretreated as described in part 2.3.1. The volume of the right hind foot was measured as the basic value before the experiment. One hour after the last intragastric administration, the right hind paw of mice received a subcutaneous injection of 1% w/v carrageenan in physiological saline (30 μL) to induce acute inflammation. Using a digital toe volume measuring instrument, the paw swelling was determined at 1, 2, 3, and 4 hours after injection. The

difference between the paws before and after treatment with carrageenan was applied to measure the degree of swelling. A proportion of edema inhibition was calculated relative to the paw volume of the negative control group to determine the anti-inflammatory activity (Sousa et al. 2018). A physiological saline injection was given subcutaneously to mice in the negative control group.

PGE_2 levels in serum

After the acetic acid writhing experiment, all mice were immediately anaesthetized with 1% isoflurane, blood was gained from the abdominal aorta, and serum was isolated for preparation. Using a mouse PGE_2 ELISA kit, the serum concentration of PGE_2 was measured according to the manufacturer's recommendations.

Separation and purification of CSP-A and CSP-B

First, CSP-A was fractionated and eluted using a DEAE-52 cellulose ion-exchange chromatography column (5 cm x 100 cm) at a flow rate of 5 mL/min with a stepwise gradient of NaCl aqueous solution (0, 0.1, and 0.2 mol/L). The carbohydrate content was determined using the phenol-sulfuric acid technique for elution curve charting (Albalasmeh et al. 2013). After that, the main polysaccharide fraction (CSP-A1) was further purified by an HW-55F gel column (5 cm × 120 cm). The above polysaccharide fraction was repeatedly purified using Sephacryl S-400 columns (3 cm × 120 cm). Two milliliters of distilled water were passed through the column each minute. The eluate was collected (5 mL/tube), concentrated and lyophilized.

As for CSP-B, it was initially isolated using a DEAE-52 cellulose column in the same way. Using a HW-40 size exclusion column and elution at a rate of 2 mL/min with distilled water, the major fraction was further purified. Pure polysaccharide was obtained by collecting, concentrating, and lyophilizing the eluted solution at the peak (5 mL/tube).

Structural characterizations of polysaccharides

Molecular weight determination

According to the method of Zhao et al. (Zhao et al. 2021), the molecular weight of compound 1 was carried out using the HPLC system (2695, Waters, Agilent Technologies Inc., USA) equipped with a 2414 refractive index detector.

Molecular weights of dextran standards ranging from 2000000 to 5250 Da were used to establish the standard curve. The data was analyzed by Empower 2 software. In addition, the molecular weight of compound 2 was relatively small, so its molecular weight was calculated by electrospray ionization time-of-flight mass spectrometry (ESI-TOF/MS).

Monosaccharide compositions determination

By ion chromatography, two compounds were analyzed for their monosaccharide compositions (Dionex ICS-5000, Thermo Fisher Scientific, US) (Xiong et al. 2015). In brief, 2 mL of trifluoroacetic acid solution (TFA, 4 M) solution was used to hydrolyze five milligrams of samples at 120°C for 3 hours. The remaining TFA in the tube was removed by nitrogen blowing, and then the residues were dissolved in ultrapure water. All samples were finally diluted to the concentration of 50μg/mL and filtered through a 0.22 μm microporous membranes for analysis.

NMR spectroscopy analysis

Nuclear magnetic resonance analysis was performed on the basis of the previous method(Hu, Xu, et al. 2022), and modified slightly. Two compounds were completely dissolved in deuteroxide (D_2O) and dried in vacuum by a rotary evaporator. The above steps were repeated twice. Compounds 1 and 2 were re-dissolved with D_2O at a concentration of 30 mg/mL, respectively. ^{1}H, ^{13}C, HSQC and HMBC NMR spectra were recorded using a Bruker AVANCE III HD spectrometer.

Anti-inflammatory activity in vitro

Compounds 1 and 2 were evaluated for anti-inflammatory activity in mouse macrophage RAW 264.7 cells in vitro. The cells were cultured overnight in a 96-well plate (100 μL/well) at a concentration of 2×10^5 cells/mL. Cell viability was determined by CCK-8 assay after 24 h of incubation with either compound at various concentrations (50, 100, 200, 400 and 800 μg/mL) (Zhu et al. 2019). A 6-well plate was inoculated with RAW264.7 cells (2×10^4 cells/well) and cultured overnight for anti-inflammatory activity testing. Compound 1 and compound 2 (10, 200, and 400 g/mL, respectively) were applied to macrophages stimulated with 500 ng/mL LPS (Liu et al. 2022). The supernatant of the cells was collected to measure the levels of nitric oxide and cytokines. In accordance with the manufacturer's instructions,

nitric oxide (NO), tumor necrosis factor alpha (TNF-a), interleukin-6 (IL-6) and interleukin-1β (IL-1β) concentrations in cell supernatant were determined using the NO assay kit and mouse ELISA kits, respectively.

Statistical analysis

The mean ± standard error (SEM.) was used to express all values. By using a one-way analysis of variance, the significant differences between the groups (n = 10) were evaluated. $P<0.01$ denoted an extremely significant difference, whereas $P<0.05$ was regarded as statistically significant.

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Table S1 Effects of extracts from Coix seed on latency time of hot plate test in mice

Group	Dose (g/kg)	Latency time (s)			
		0 min	30 min	60 min	90 min
NC	-	17.96±0.26	18.31±0.30	18.94±0.22	19.17±0.28
AC	0.25	18.48±0.93	33.56±0.26**	36.87±0.53**	34.62±0.54**
CSE	0.60	17.99±0.35	20.06±0.78	20.32±0.79	20.64±0.74
CSP-A	0.60	18.63±0.41	26.78±0.29**	37.18±0.79**	32.04±0.60**
CSP-B	0.60	18.72±0.35	21.47±0.78	24.03±0.79	21.75±0.74

Values are expressed as mean ± SEM (n = 10). **P<0.01, compared to the negative control group.

Table S2 ^{1}H and ^{13}C NMR chemical shifts of compound 2

Residue		^{1}H and ^{13}C chemical shifts (ppm)					
		1	2	3	4	5	6
β-D-Frup (2→	δ_H	3.63		4.09	4.02	3.77	3.67
	δ_C	65.18	106.45	78.61	75.36	83.99	63.84
→1)-α-D-Glcp (6	δ_H	5.29	3.51	3.68	3.48	3.97	4.06
	δ_C	94.75	74.06	76.79	71.99	72.31	69.18
	δ_H	4.92	3.50	3.67	3.74	4.00	4.07
	δ_C	100.97	73.90	76.79	71.89	72.14	69.08
	δ_H	4.92	3.49	3.67	3.76	3.96	3.84
	δ_C	100.69	73.62	76.69	71.36	72.13	68.47
	δ_H	4.92	3.48	3.62	3.74	3.96	3.89
	δ_C	100.65	73.59	76.64	71.09	72.13	68.38
	δ_H	4.92	3.48	3.62	3.74	3.99	3.90
	δ_C	100.61	73.59	76.64	70.93	72.06	68.27
→1)-α-D-Glcp (4	δ_H	5.17	3.46	3.71	3.42	3.81	3.85
	δ_C	94.85	72.55	75.68	78.93	72.02	63.84
→1)-α-D-Glcp	δ_H	4.60	3.48	3.68	3.45	3.99	3.97
	δ_C	98.73	71.99	76.71	71.40	72.06	64.02

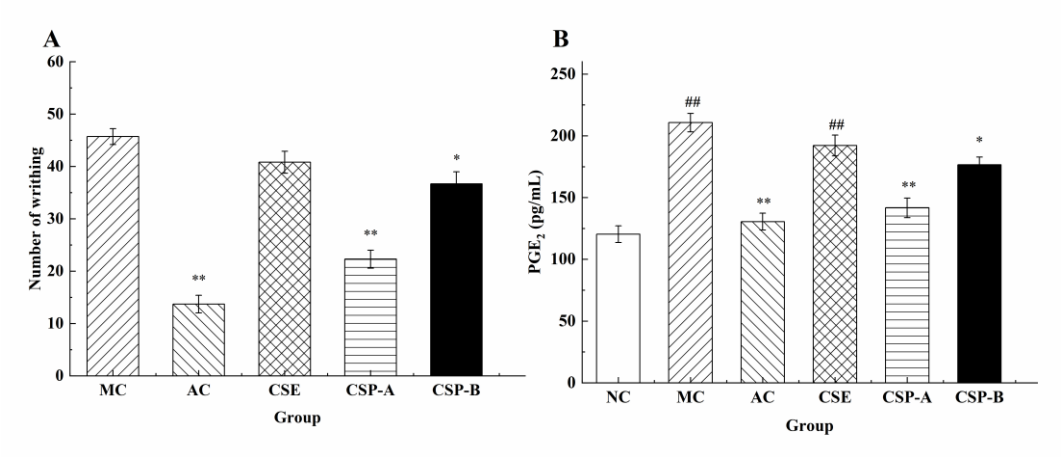


Figure S1 (A) The impact of extracts on the acetic acid-induced torsional response in mice; (B) The concentration of PGE_2 in serum of mice.NC, negative control group; MC, model group; AC, positive control group; CSE, ethanol extract group, CSP-A, CSP-A group; CSP-B, CSP-B group. ##P<0.01, compared to the negative control group. *P<0.05, **P<0.01, compared to the model group.

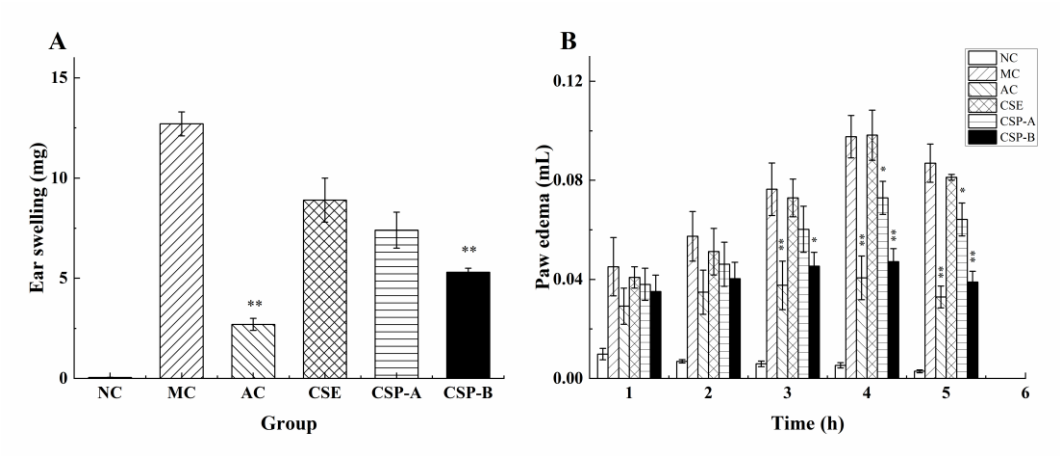


Figure S2 Anti-inflammatory activity of Coix seed extract (A) The effects of extracts from Coix seed on xylene-induced ear swelling in mice. (B) The effects of extracts from Coix seed on carrageenan-induced paw edema in mice. NC, negative control group; MC, model group; AC, positive control group; CSE, ethanol extract group, CSP-A, CSP-A group; CSP-B, CSP-B group. *P<0.05, **P<0.01, compared to the model group.

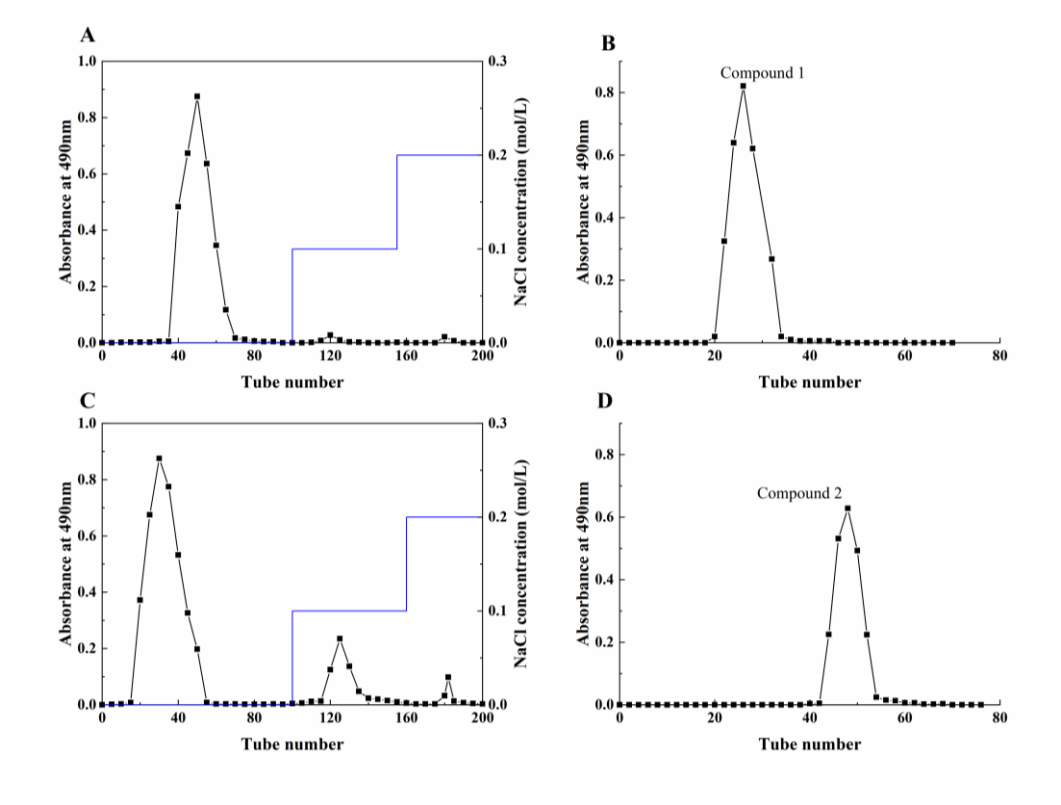


Figure S3 Elution curves. (A) CSP-A on DEAE-52 cellulose column; (B) CSP-A1 on Sephacryl S-400 column; (C) CSP-B on DEAE-52 cellulose column; (D) CSP-B1 on HW-40 size exclusion column.

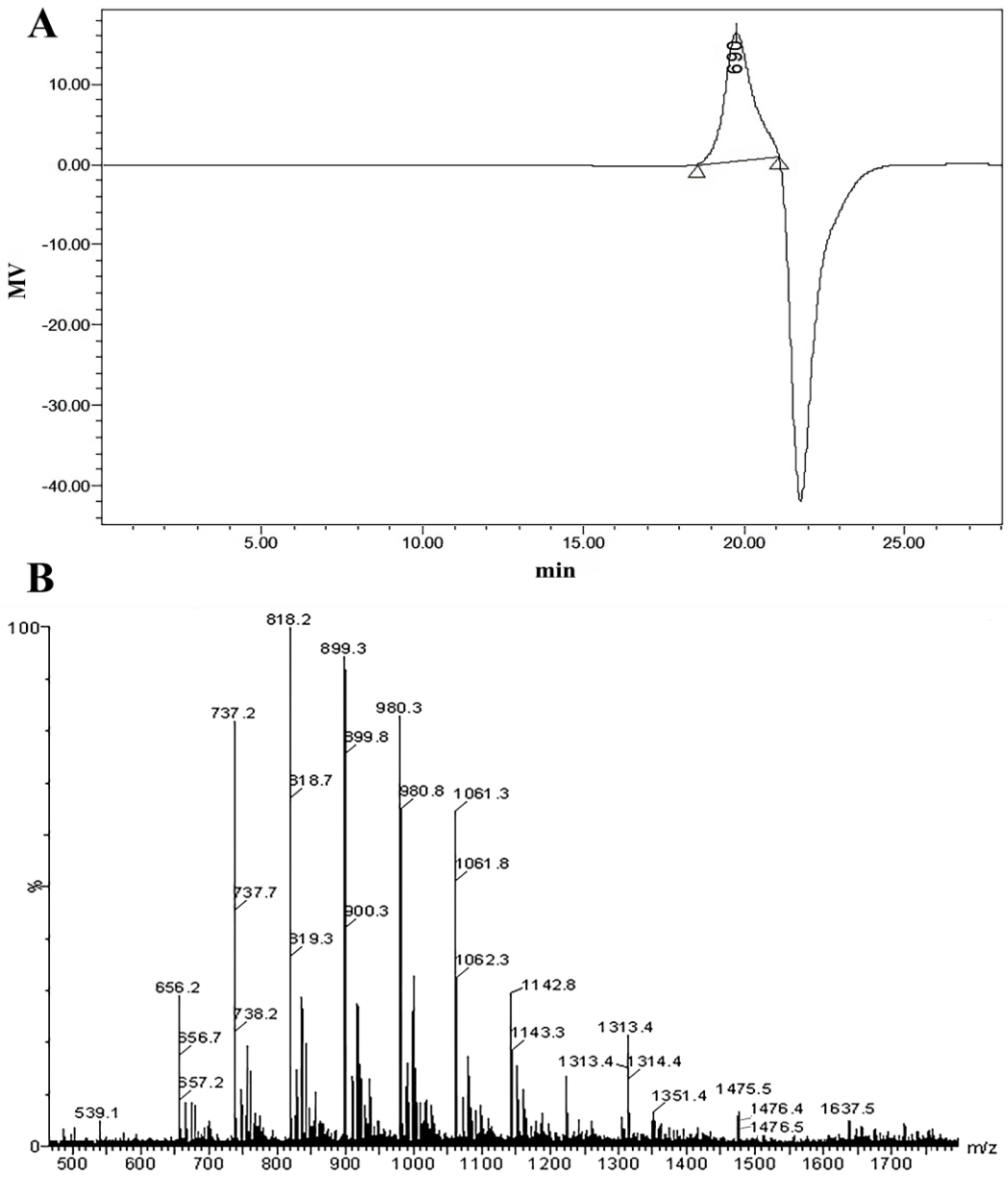


Figure S4. Molecular weight determination of compound 1 and compound 2. (A) Chromatogram of compound 1 by HPLC; (B) ESI-TOF/MS spectrum of compound 2.

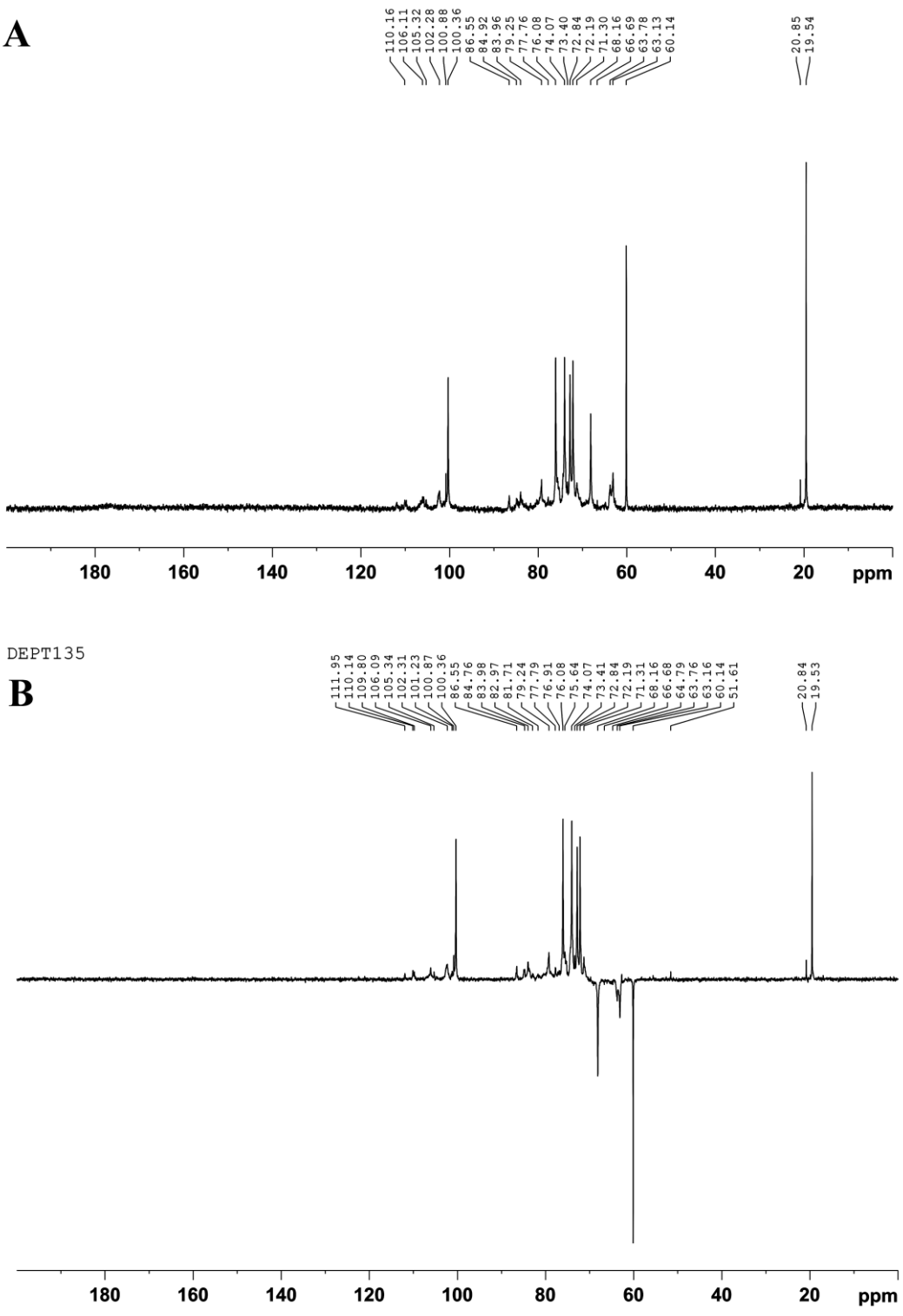
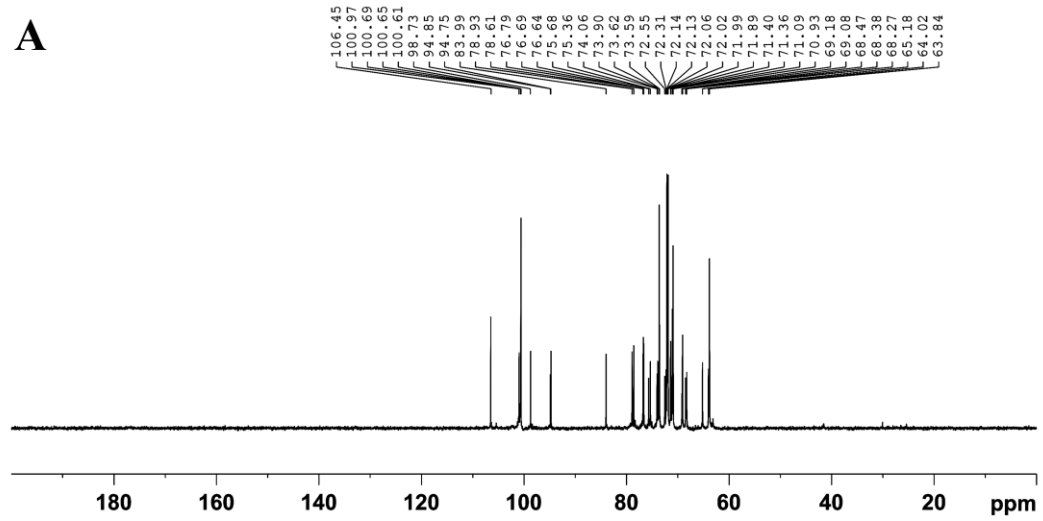


Figure S5. Structural characteristics of compound 1. (A) ^{13}C NMR spectrum; (B) DEPT 135° spectrum of compound 1.

A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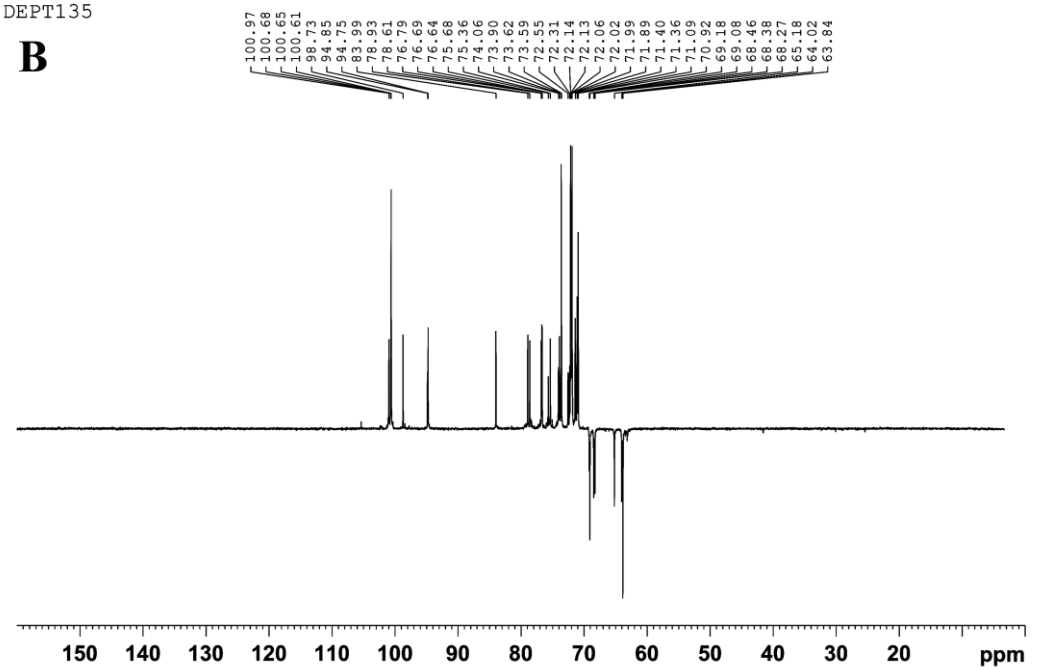
B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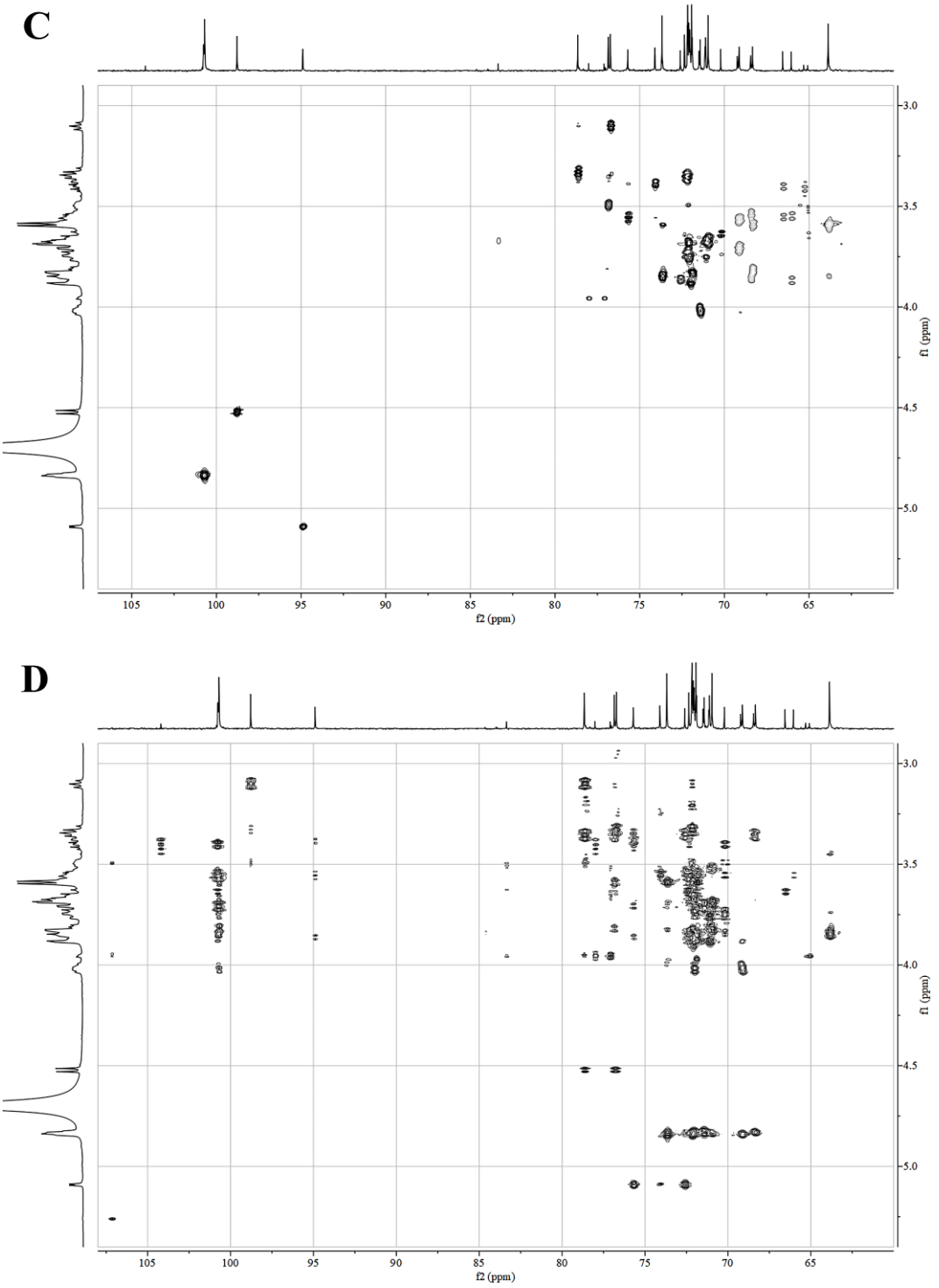

Figure S6. Structural characteristics of compound 2. (A) ^{13}C NMR spectrum; (B) DEPT 135° spectrum; (C) HSQC spectrum and (D) HMBC spectrum of compound 2.

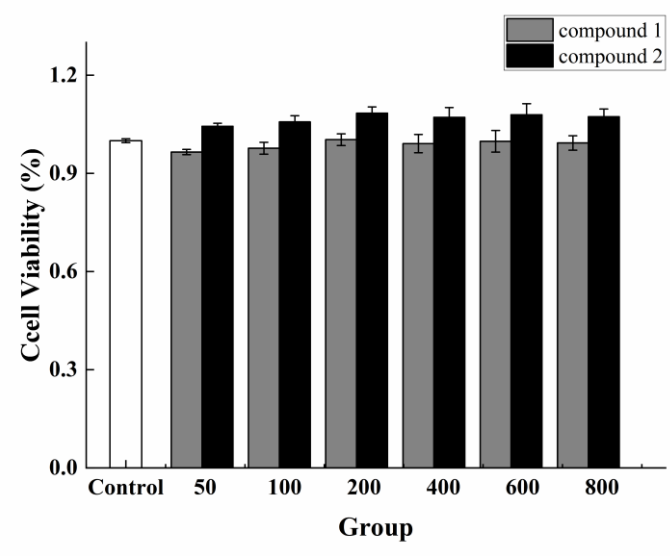

Figure S7 The effects of two compounds in Coix seed (compounds 1 and 2) on cell viability.

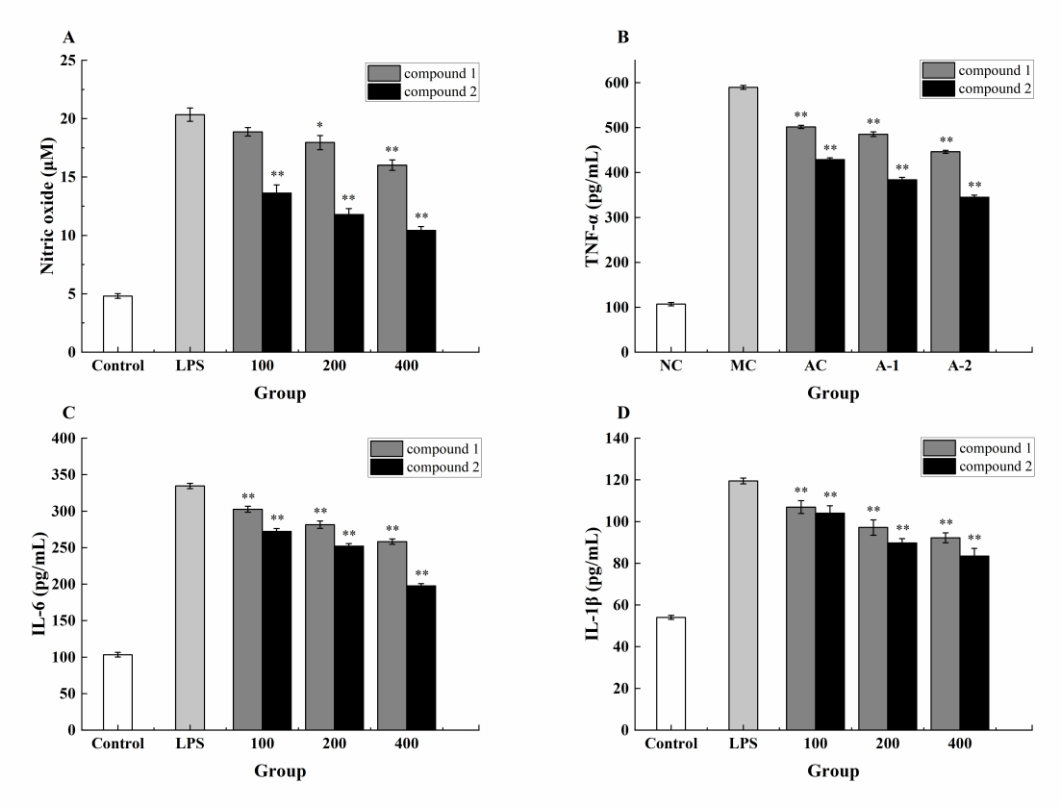

Figure S8. The effects of two compounds on LPS-induced inflammation in RAW264.7 cells. (A) NO production, (B) TNF-α production, (C) IL-6 production, (D) IL-1β production. Data are expressed as the means ± SEM (n = 6). *P<0.05, **P<0.01, compared with LPS-treated group.