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Extraction, isolation, immunoregulatory activity, and characterization of *Alpiniae oxyphyllae* fructus polysaccharides

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ABSTRACT

For further applications of *Alpiniae oxyphyllae* fructus in modern clinical medicine, *Alpiniae oxyphyllae* fructus polysaccharide (AOFP) was studied in the present work. The extraction conditions of AOFP were optimized by the response surface method with a Box-Behnken design. The maximum extraction rate of AOFP was 3.18%. An anion-exchange DEAE-52 cellulose column and a Sephadex G-100 gel column were used to isolate the AOFP, and three polysaccharides (AOFP1, AOFP2, AOFP3) were obtained. All three polysaccharides possessed immunoregulatory activity, but the effects of AOFP1 were greater than the other two polysaccharides. AOFP1 significantly stimulated Th1- and Th2-type immune responses and specific immune responses. Meanwhile, the characterization of AOFP1 was studied. AOFP1 was composed of arabinose, galactose, glucose, xylose, mannose, galacturonic acid, and glucuronic acid at a molar ratio of 16.46:12.7:4.9:17.11:4.35:6.52:6 with an average molecular weight of 43.4 kDa. These results suggest that AOFP1 can be developed as a natural immunomodulatory drug.

1. Introduction

Alpiniae oxyphyllae fructus, which is the dry ripe fruit of *Alpinia oxyphylla* Miq. has a long history of application in traditional clinical Chinese medicine. It is widely used for treatment of emesis, diarrhea, frequent urination, and spermatorrhea [1]. Additionally, modern medical research indicates that *Alpiniae oxyphyllae* fructus also works on Alzheimer's disease [1] and chronic kidney injury [2]. However, there is little research on the active ingredients of *Alpiniae oxyphyllae* fructus. Therefore, research in this field is meaningful and necessary for the further application of *Alpiniae oxyphyllae* fructus in modern clinical medicine.

Polysaccharides, one of the main active ingredients in traditional Chinese medicine, have a wide range of biological activities. They play important roles in the regulation of the immune system [3,4], tumor growth [5], virus multiplication [6], and oxidative balance [7]. Recently, Han et al. [8] reported that the yield of *Alpiniae oxyphyllae* fructus polysaccharide (AOFP) was 1.78%, which indicated that polysaccharide was one of the main effective components of *Alpiniae oxyphyllae* fructus. However, the yield of *Alpiniae oxyphyllae* fructus polysaccharides from this study is still relatively low. Moreover, the isolation and characterization of AOFP [8] were less accurate. Therefore, the extraction, composition and structure of AOFP was studied in this paper.

Sun, Wang & Zhou [9] declared that *Porphyridium cruentum* polysaccharides strengthened macrophage activity. Hou et al. [10] concluded that selenylation of lily polysaccharide promoted lymphocyte proliferation. We also reported that the activities of T and B lymphocytes were enhanced by bush sophora root polysaccharide in our previous study [11]. In summary, polysaccharides from traditional Chinese medicine often show immunoregulatory activities. Here, we investigated whether AOFP possessed immunoregulatory activity, including evaluation of the regulation of T and B lymphocytes.

In the present study, polysaccharides were extracted from *Alpinia oxyphylla* Miq. after the optimal extraction conditions were obtained by the response surface method (RSM). Next, the polysaccharides were purified with an anion-exchange DEAE-52 column and Sephadex G-100 gel column. Then, the immunomodulatory activity, including lymphocyte proliferation, Th1- and Th2-type immune responses, and the specific immune response of polysaccharides were analyzed, and the chemical characterization was detected by gel permeation

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chromatography (GPC)-RI-MALS, high performance anion exchange chromatography (HPAEC)-PAD, infrared spectroscopy (FT-IR) and nuclear magnetic resonance spectroscopy (NMR).

2. Materials and methods

2.1. Reagents

Alpiniae oxyphyllae fructus was purchased from Lvfuxian Agricultural Development Co., Ltd. (Danzhou, China). Cellulose DEAE-52 and Sephadex G-100 were purchased from GE Healthcare (Fairfield, USA). Chloroform and N-butyl alcohol were purchased from Nanjing Chemical Reagent CO., Ltd. (Nanjing, China). RPMI-1640 and fetal bovine serum albumin (BSA) were purchased from Gibco Life Technologies (Carlsbad, USA). Penicillin and streptomycin were purchased from HyClone (Logan, USA). All standard monosaccharides (fucose, arabinose, galactose, glucose, xylose, mannose, fructose, ribose, galacturonic acid and glucuronic acid), red cell lysis solution, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), phytohaemagglutinin (PHA), lipopolysaccharide (LPS), and ovalbumin (OVA) were purchased from Sigma-Aldrich (St. Louis, USA). Mouse IL-2, IL-4, IL-6, and IFN-γ ELISA kits were purchased from Beyotime Institute of Biotechnology Co., Ltd. (Shanghai, China). The mouse OVA-sIgG ELISA Kit was purchased from Yifeixue Biotechnology Co., Ltd. (Nanjing, China).

2.2. Response surface method

2.2.1. Extraction of AOFP

According to the method of water extraction and alcohol precipitation, the AOFPs were extracted under the conditions of the designed decoction temperature, decoction duration, ratio of water to *Alpiniae oxyphyllae* fructus, and ethanol concentration. Then, crude polysaccharide precipitates were desiccated (60 °C, 5 h) after centrifugation (3500 r, 15 min). Next, the polysaccharide content was determined by the phenol-sulfuric acid method [12].

2.2.2. Design of single-factor tests

The influence of four single factors (decoction temperature, decoction duration, the ratio of water to *Alpiniae oxyphyllae* fructus, and ethanol concentration) mentioned in Section 2.2.1 on the extraction rate of AOFP was determined. The contributing factors were adopted in the next experiment.

2.2.3. Optimization on extraction conditions of AOFP

The extraction conditions of AOFP were optimized by the Box–Behnken design (BBD) in the RSM, containing 27 experiments. The four variables were decoction temperature (A), decoction duration (B), ethanol concentration (C), and ratio of water to *Alpiniae oxyphyllae* fructus (D). As Table 1 shows, the above four factors were encoded at three levels (−1, 0, 1). The experimental data were analyzed by the following quadratic polynomial regression equation:

$$Y = f_0 + \sum_{i=1}^{4} f_i x_i + \sum_{i=1}^{4} f_{ii} x_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} f_{ij} x_i x_j$$

In the equation, the extraction rate of the polysaccharide is the response value (Y). *Xi* and *Xj* are independent variables; f_0 is the intercept; and f_i, f_j, and f_{ij} represent regression coefficients of linear, quadratic and interactive terms, respectively. The number of test variables was 4.

The coefficients of the linear, quadratic, and interaction terms were analyzed by design-expert software (version 8.0.6, Stat-Ease, Inc., Minneapolis, USA). The significance of each coefficient was analyzed by analysis of variance (ANOVA). The influence of each independent variable on the response value was reflected by the two-dimensional contour charts and three-dimensional response surface charts. The determination coefficient (R^2) was used to evaluate the degree of closeness of the polynomial equation to the response value.

Table 1
Variables, codes, and levels of BBD design.

Variable	Coded	Levels and range		
		−1	0	1
Decoction temperature (°C)	A	80	90	100
Decoction duration (min)	B	45	60	75
Ethanol concentration (%)	C	70	75	80
Ratio of water to *Alpiniae oxyphyllae* fructus (mL:g)	D	10	12	14

2.3. Purification and isolation of AOFP

Crude AOFP was obtained by the water extraction and alcohol precipitation method according to the optimal extraction conditions of the RSM. The crude polysaccharide solution was mixed with Sevage reagent (chloroform/N-butyl alcohol = 4/1, v/v) according to the volume ratio of 4:1 to remove the proteins [13]. This treatment was repeated until no denatured proteins were present in the polysaccharide liquid. Then, the deproteinized polysaccharides were dissolved in distilled water and applied to a DEAE-52 cellulose column (2.6 cm × 30 cm) using distilled water, and 0.2, 0.4, and 0.6 M NaCl solutions at a flow rate of 0.5 mL/min were used as the eluent. The eluate was automatically collected at 20 min intervals by a fraction collector (BS-100A; Shanghai Huxi, China). The fractions were collected, and the polysaccharide content was detected by the phenol-sulfuric acid method [12]. The obtained polysaccharide fractions were further purified with a Sephadex G-100 column (2.6 cm × 90 cm), and the column was eluted with distilled water at a flow rate of 0.5 mL/min. The collection of eluate was the same as described above. The purified polysaccharide fractions were collected, dialyzed, concentrated, and freeze-dried.

2.4. Analysis of the immunomodulatory activity of AOFP

2.4.1. Animals

Male ICR mice (6 weeks old and weighing 18.0–22.0 g) were purchased from Sichuan Laboratory Animal Center (SLAC) Co., Ltd. (Sichuan, China). The mice were placed in plastic cages containing wood chips at room temperature. All experiments were approved and monitored by the Animal Care and Use Committee of Animal Science and Technology College of Hainan University.

2.4.2. Cytotoxicity test

Mouse splenic lymphocytes were isolated according to the method of Huang et al. [14]. In brief, the spleen was obtained from an ICR mouse and then gently pressed on a stainless steel sieve (200 mesh) with a syringe plunger. Then, the supernatant was removed from the cells after centrifugation (1000 r, 5 min, 4 °C). Afterwards, red cell lysis solution was added to the cells followed by centrifugation again. Finally, the cells were washed twice with RPMI-1640. The purified mouse lymphocytes (2.5×10^6/mL) were cultured in 96-well plates in 100 μL per well of RPMI-1640 with 10% (*V*/V) BSA, 100 IU/mL penicillin, and 100 IU/mL streptomycin. Then, 100 μL of RPMI-1640 (cell control) or various concentrations of AOFP1, AOFP2, and AOFP3 (0.0625–8 mg/mL) were added to the 96-well plates, with each concentration repeated 3 times. After incubation (37 °C, 5% CO_2) for 48 h, the cytoactivity of the splenic lymphocytes was detected by MTT assay [15].

2.4.3. Lymphocyte proliferation assay

According to the results of the cytotoxicity test, three concentrations (4 mg/mL, 2 mg/mL, and 1 mg/mL) of each polysaccharide were applied in the lymphocyte proliferation test. Eighty microliters of splenic lymphocytes (2.5×10^6/mL) were added to 96-well plates [16]. Then, 100 μL of polysaccharide and 20 μL of PHA/LPS were added to the polysaccharide

group, 100 μL of RPMI-1640 and 20 μL of PHA/LPS were added to the PHA/LPS group, and 120 μL of RPMI-1640 was added into the cell control group, with each treatment being repeated 3 times. After incubation (37 °C, 5% CO_2) for 48 h, the proliferation of lymphocytes was determined by MTT assay [15]. The optimal AOFP with lymphocyte proliferation stimulation activity was screened out and applied in the next experiment.

2.4.4. Th1- and Th2-type immune responses

Mouse splenic lymphocytes were obtained as described in Section 2.4.2. One hundred sixty microliters of purified lymphocytes (2.5×10^6/mL) were cultured in 24-well plates. After that, 200 μL of polysaccharide and 40 μL of PHA were added to the polysaccharide group, 200 μL of RPMI-1640 and 40 μL of PHA were added to the PHA group, 240 μL of RPMI-1640 was added to the cell control group, with each treatment being repeated 3 times. After incubation (37 °C, 5% CO_2) for 48 h, the levels of IL-2, IFN-γ, IL-4, and IL-6 in the supernatant were tested by the corresponding ELISA kits [17].

2.4.5. Determination of OVA-specific sIgG

ICR mice (6 weeks old) were intraperitoneally injected with OVA once a week. Two weeks later, mouse splenic lymphocytes were isolated and treated according to Section 2.4.4, where the PHA was replaced by LPS. Furthermore, OVA solution was added to each well. The supernatant was collected after incubation for 48 h. Then, OVA-specific sIgG was detected [3].

2.5. Characterization of the polysaccharide

2.5.1. Molecular weight determination

The test polysaccharides (5 mg) were dissolved in 0.1 M $NaNO_3$ and heated (100 °C). Then, 100 μL of polysaccharide supernatant was sampled after centrifugation. Next, the solution was detected by gel permeation chromatography with a refractive index (RI) detector and MALS (DAWN HELEOS II, USA). The mobile phase was 0.1 M $NaNO_3$ with a flow rate of 0.4 mL/min. Finally, the results of polysaccharide molecular weight were determined with ASTRA 6.1 software.

2.5.2. Monosaccharide composition

The monosaccharide composition of the polysaccharide fraction was analyzed with HPAEC equipped with a Dionex™ CarboPac™ PA20 analytical column (250 × 2 mm) with pulsed amperometric detection. Briefly, the polysaccharide sample (5 mg) was dissolved in TFA solution (1 mL) at 121 °C for 2 h. Then, the polysaccharide acidolysis solution was dried with a termovap sample concentrator. After that, methanol was added to the dried polysaccharide sample followed by blow drying. Each process was repeated 3 times. Finally, the polysaccharide sample was dissolved in sterile water and transferred to a chromatographic flask for testing.

Fucose, arabinose, galactose, glucose, xylose, mannose, fructose, ribose, galacturonic acid and glucuronic acid (100 mg each) were successively added into sterile water and quantitatively dissolved in a 10 mL volumetric flask to prepare a mixed standard solution. Then, standard working solutions were obtained from the mixed standard solution that was gradient diluted to 1/100, 5/100, 10/1000, 20/1000, 30/100,

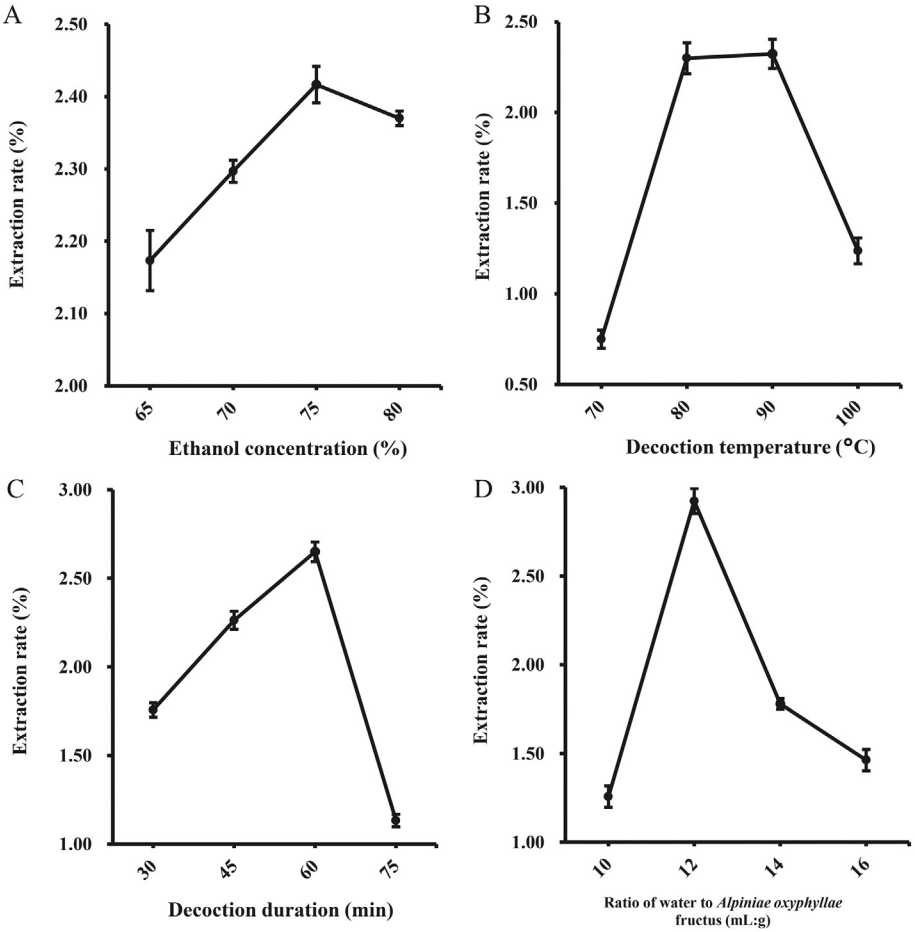

Fig. 1. Effects of the four single factors on the extraction rate of AOFP. The values presented are the mean ± S.D. ($n = 3$ in each group).

40/100, 50/100 and 60/100. Finally, the diluted solutions was transferred to the chromatographic bottle for testing.

The test polysaccharide fractions and mixed standards solution were eluted by a gradient of mobile phases (A) distilled H_2O, (B) 200 mM NaOH and (C) 200 mM NaOH/500 mM NaAC (A:B:C was 97.5:2:0 from 0 to 25 min, 97.5:2:0 from 25 to 25.1 min, 77.5:2.5:20 from 25.1–40 min, 77.5:2.5:20 from 40 to 40.1 min, 0:0:100 from 40.1–50 min, 0:0:100 from 50 to 50.1 min, and 97.5:2.5:0 from 50.1–60 min, at a flow rate of 0.5 mL/min) with an injection volume of 25 μL.

2.5.3. FT-IR analysis

The infrared spectrum of the polysaccharide was determined by the KBr pressed-tablet technique [15]. Briefly, purified polysaccharides (2 mg) were ground with dry KBr powder and pressed into a tablet. Then, the tablet was measured with a Tensor 27 FT-IR Spectrometer (Bruker, Karlsruhe, Germany) from 4000 to 400 cm^{-1}.

2.5.4. NMR analysis

The complex structure of the polysaccharides was analyzed by NMR. The polysaccharide fractions were freeze-dried and dissolved in D_2O. Then, the ^1H, ^{13}C, and HSQC NMR spectra were recorded on a Bruker AV-600 spectrometer at 25 °C. The experimental results were obtained using MestReNova 12.0.0 software analysis.

3. Results

3.1. Extraction of AOFP

3.1.1. Single-factor experiments

Fig. 1 shows the effects of four single factors (decoction temperature, decoction duration, ratio of water to *Alpiniae oxyphyllae* fructus, and ethanol concentration) on the extraction rate of AOFP. When three factors were controlled at the same level, all four factors influenced the extraction rate of AOFP. A better AOFP extraction rate was obtained when the ethanol concentration was 75% (Fig. 1A), the decoction temperature was 90 °C (Fig. 1B), the decoction duration was 60 min (Fig. 1C), or the ratio of water to *Alpiniae oxyphyllae* fructus was 12:1 (Fig. 1D). Therefore, these factors and conditions were selected in the subsequent RSM experiments.

3.1.2. Fitting the model

BBD, an RSM design method, is suitable for experimental conditions with fewer than 5 factors and 3 levels [18]. Here, 27 experiments with 3 levels and 4 factors were carried out (Table 2). The results showed that the minimum extraction rate of polysaccharide was 0.63%, while the maximum polysaccharide extraction rate was 3.23% at the conditions of A = 90, B = 60, C = 75, and D = 12. According to the results in Table 2, the response variables (extraction rate of polysaccharide) and test coefficients were fitted by the following second-order polynomial equation:

$$Y = 3.22 + 0.031A - 0.084B + 0.073C - 0.12D - 0.1AB + 0.02AC - 0.027AD - 0.097BC - 0.03BD + 0.11CD - 0.31A^2 - 0.91B^2 - 0.54C^2 - 1.5D^2$$

Table 3 shows the ANOVA results for the response surface quadratic model. The *P*-value for the model and lack of fit were <0.0001 and 0.057, respectively. Meanwhile, the R^2 and R^2_{Adj} values were 0.9935 and 0.9860, respectively. These results indicated that this model can be used to predict the extraction rate of polysaccharides with high correlation within the designed range. Additionally, both the linear coefficient and the quadratic coefficient were statistically significant, as the *P*-values of B, C, D, A^2, B^2, C^2, and D^2 were <0.05. Furthermore, three interaction terms (AB, BC and CD) were statistically significant ($P < .05$).

Table 2
The design and results of the Box–Behnken experiments.

No.	A (°C)	B (min)	C (%)	D (mL/g)	Response: Y (%)
1	100	60	75	10	1.50
2	100	60	75	14	1.24
3	90	75	75	14	0.63
4	90	75	70	12	1.68
5	90	45	70	12	1.68
6	80	60	75	14	1.30
7	80	60	80	12	2.50
8	100	60	80	12	2.48
9	80	60	75	10	1.45
10	90	60	80	14	1.23
11	100	75	75	12	1.93
12	100	45	75	12	2.30
13	90	60	70	10	1.30
14	80	75	75	12	1.88
15	90	45	75	10	1.02
16	90	45	80	12	1.98
17	90	60	75	12	3.23
18	80	60	70	12	2.39
19	90	75	80	12	1.59
20	90	60	70	14	0.83
21	100	60	70	12	2.29
22	90	60	75	12	3.23
23	80	45	75	12	1.85
24	90	45	75	14	0.83
25	90	75	75	10	0.94
26	90	60	80	10	1.27
27	90	60	75	12	3.19

3.1.3. Optimization of polysaccharide extraction conditions

The AOFP extraction rate affected by the four factors is depicted in Fig. 2 through two-dimensional contour charts and three-dimensional response surface charts, which showed the effects of two factors on response when the other two were at the level of zero. The results indicated that each factor exhibited a similar effect on the AOFP extraction rate: a trend to first increase and then decrease. As Fig. 2A shows, when the decoction temperature was approximately 90 °C, the AOFP extraction rate was the highest; when the decoction duration was approximately 60 min, the AOFP extraction rate was the highest. Fig. 2B shows that the AOFP extraction rate was comparatively higher when the ethanol concentration and extraction duration were 75% and 60 min, respectively. Additionally, Fig. 2C indicates that when the ratio of water to *Alpiniae oxyphyllae* fructus was 12:1 and the ethanol concentration was 75%, the AOFP extraction rate was the highest.

Table 3
ANOVA for the response surface quadratic model.

Source	Sum of squares	df	Mean square	F value	Prob > F
Model	14.06	14	1	131.82	<0.0001
A	0.011	1	0.011	1.5	0.2445
B	0.085	1	0.085	11.16	0.0059
C	0.065	1	0.065	8.47	0.0131
D	0.17	1	0.17	22.06	0.0005
AB	0.04	1	0.04	5.25	0.0408
AC	0.0016	1	0.0016	0.21	0.6549
AD	0.003025	1	0.003025	0.4	0.5404
BC	0.038	1	0.038	4.99	0.0452
BD	0.0036	1	0.0036	0.47	0.5048
CD	0.046	1	0.046	6.07	0.0298
A^2	0.5	1	0.5	65.86	<0.0001
B^2	4.38	1	4.38	575.66	<0.0001
C^2	1.57	1	1.57	206.41	<0.0001
D^2	12.05	1	12.05	1581.77	<0.0001
Residual	0.091	12	0.007616		
Lack of Fit	0.09	10	0.009033	16.94	0.057
Pure Error	0.001067	2	0.0005333		
Cor Total	14.15	26			

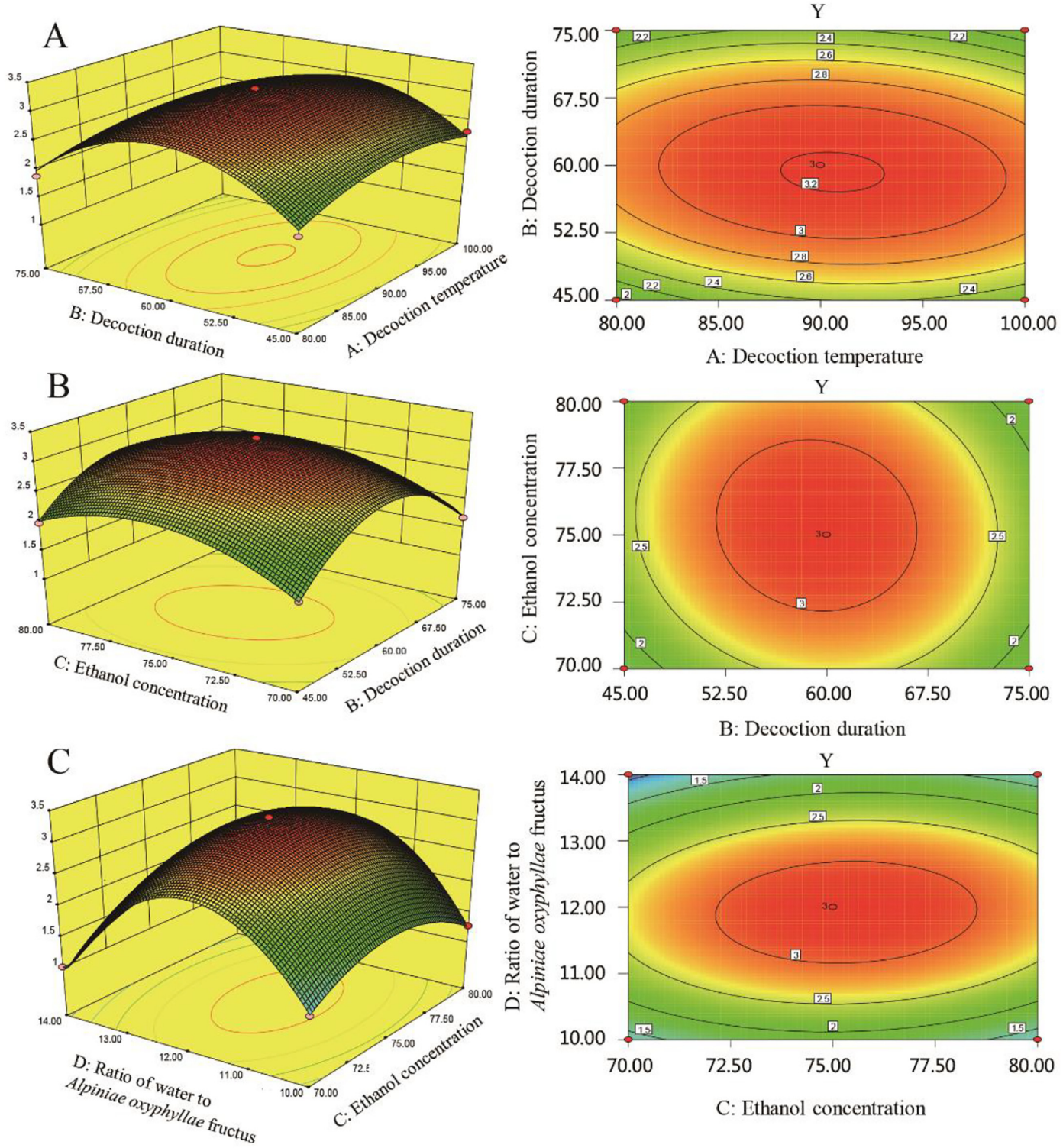

Fig. 2. Three-dimensional response surface charts and two-dimensional contour charts of the interactive effects of decoction temperature and decoction duration (A), decoction duration and ethanol concentration (B), and ethanol concentration and ratio of water to *Alpiniae oxyphyllae* fructus (C) on the AOFP extraction rate.

The optimal AOFP extraction rate analyzed by Design-Expert software was a decoction temperature of 89.59 °C, a decoction duration of 61.08 min, an ethanol concentration of 75.12%, and a ratio of water to *Alpiniae oxyphyllae* fructus of 11.76:1. Moreover, according to the results in Table 3 and Fig. 2, the ratio of water to *Alpiniae oxyphyllae* fructus was the most significant factor that affected the AOFP extraction rate, followed by the decoction duration, ethanol concentration, and decoction temperature.

3.1.4. Verification of the RSM

Theoretically, the extraction rate of AOFP was 3.23% under the optimal extraction conditions (decoction temperature of 89.59 °C, decoction

duration of 61.08 min, ratio of water to *Alpiniae oxyphyllae* fructus of 11.76:1, and ethanol concentration of 75.12%) according to the RSM model. To validate the reliability of the RSM model, the extraction process was converted: the decoction temperature was 90 °C, the decoction duration was 60 min, the ratio of water to *Alpiniae oxyphyllae* fructus was 12:1, and the ethanol concentration was 75%. The results showed that the extraction rate of AOFP was consistent with the predicted value, which indicated that the RSM was accurate in the optimization of the polysaccharide extraction process.

3.2. Purification and isolation of AOFP

The deproteinized AOFP was first separated with a DEAE-52 cellulose column using distilled water, 0.2, 0.4, and 0.6 M NaCl solutions (Fig. 3A). Three fractions were clearly separated and named AOFP1, AOFP2, and AOFP3, respectively. Then, AOFP1 (Fig. 3B), AOFP2 (Fig. 3C), and AOFP3 (Fig. 3D) were further purified by a Sephadex G-100 column and eluted with distilled water. The results showed that there was only one symmetrically sharp peak in each fraction. Therefore, the purified AOFP1, AOFP2, and AOFP3 were eventually obtained.

3.3. Immunomodulatory activity of AOFP

3.3.1. Toxicity of the polysaccharides

Fig. 4A shows the toxicity of the polysaccharides on mouse splenic lymphocytes. The cell viability of the three polysaccharides at all tested concentrations were >100%, which meant that AOFP1, AOFP2, and AOFP3 possessed mouse splenic lymphocyte promoting abilities. However, the cell viabilities first increased and then decreased with increasing polysaccharide concentrations. This result indicated that AOFP1 possessed toxicity when its concentration was >6000 μg/mL, while AOFP2 and AOFP3 possessed toxicity when their concentrations were >8000 μg/mL. Therefore, concentrations of 4000, 2000, and 1000 μg/mL were chosen for the next experiments.

3.3.2. Effects of the polysaccharides on mouse splenic lymphocyte proliferation

As shown in Fig. 4B and C, the A_{570} values of the AOFP1, AOFP2, and AOFP3 groups were higher than those of the cell control and PHA/LPS control groups, suggesting that all three polysaccharides promoted the proliferation of mouse splenic lymphocytes. Additionally, the mouse splenic lymphocyte proliferation promotion ability of AOFP1 was relatively better than the other two fractions. Therefore, we selected AOFP1 as the research object in the following experiment.

3.3.3. Effects of AOFP1 on Th1- and Th2-type immune responses

Fig. 4D–G depicts the effect of AOFP1 on Th1- and Th2-type immune responses. The influence of AOFP1 on IL-2, IFN-γ, IL-4, and IL-6 was observed in a concentration dependent. IL-2 (Fig. 4D) and IFN-γ (Fig. 4E) secretion in the AOFP1 group were significantly ($P < .05$) higher than those in the cell control and PHA control groups, which indicated that AOFP1 greatly improved the Th1-type immune response. Similarly, compared with the cell control and PHA control, the IL-4 (Fig. 4F) and IL-6 (Fig. 4G) levels were significantly higher after AOFP1 treatment ($P < .05$). This finding proved the positive impact of AOFP1 on the Th2-type immune response.

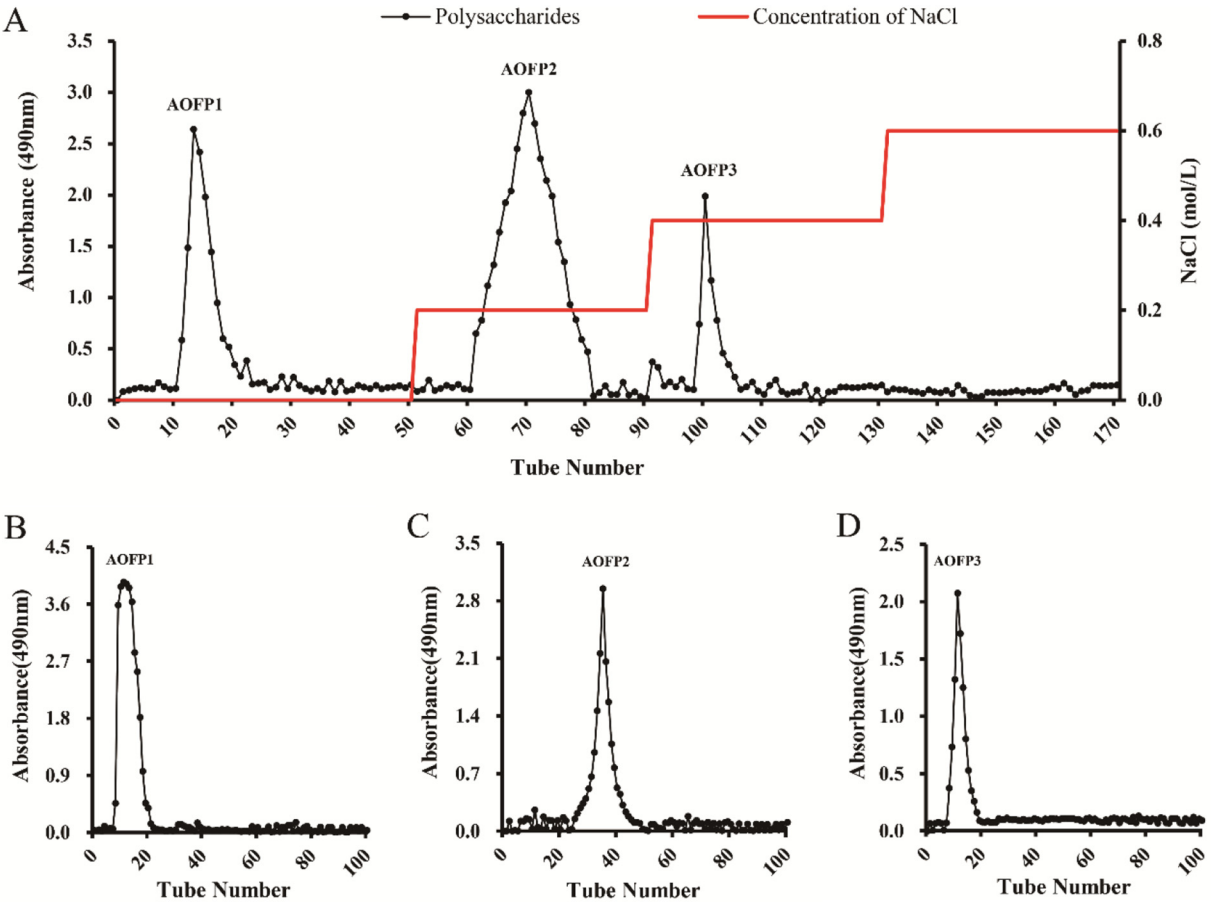

Fig. 3. Purification and isolation of *Alpiniae oxyphyllae* fructus polysaccharide (AOFP). The deproteinized AOFP was separated by a DEAE-52 cellulose column (A) and Sephadex G-100 column (B, C, and D).

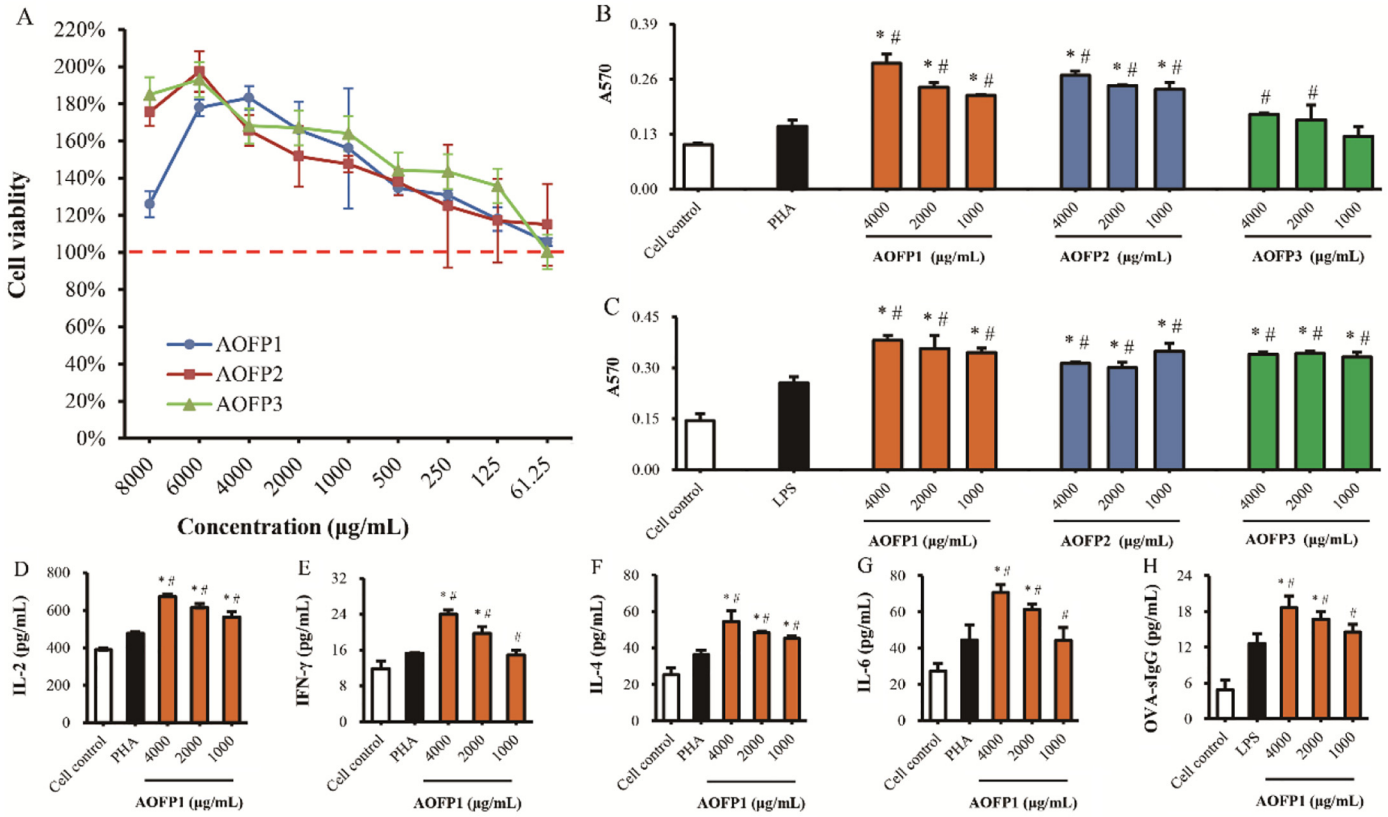

Fig. 4. Immunomodulatory activity of the AOFP. After the toxicity test of polysaccharides on mouse splenic lymphocytes (A), the effects on lymphocyte proliferation (B and C) were tested. Additionally, The effects of AOFP1 on the levels of IL-2 (D), IFN-γ (E), IL-4 (F), IL-6 (G), and OVA-sIgG (H) were also determined. The values presented are the mean ± S.D. (n = 3 in each group). $^{\#}P < .05$, compared with the cell control; $^{*}P < .05$, compared with the PHA/LPS control.

3.3.4. Effects of AOFP1 on the specific immune response

The effects of AOFP1 on the specific immune response is shown in Fig. 4H. AOFP1 promoted the production of OVA-specific sIgG in a concentration-dependent manner. Moreover, compared with the cell control and LPS control, the OVA-specific sIgG level was significantly ($P < .05$) increased after treatment with AOFP1. The above results illustrated that AOFP1 effectively improved the specific immune response.

3.4. Characterization of the polysaccharide

3.4.1. Molecular weight determination

AOFP1 showed a single and symmetrical wide peak in the GPC chromatogram (Fig. 5A), which proved that it was a relatively homogeneous polysaccharide. The average molecular weight of AOFP1 was 43.4 kDa, and its retention time was 20 min by GPC-RI-MALS analysis.

3.4.2. Monosaccharide composition

The monosaccharide composition of the polysaccharide was analyzed by HPAEC-PAD and compared with the retention time of the monosaccharide standards (Fig. 5B). The results indicated that AOFP1 was composed of arabinose, galactose, glucose, xylose, mannose, galacturonic acid, and glucuronic acid at a molar ratio of 16.46:12.7:4.9:17.11:4.35:6.52:6.

3.4.3. FT-IR analysis

The IR spectrum of AOFP1 is shown in Fig. 5C. A strong and broadly stretched absorption peak at 3431.22 cm^{-1} was attributed to the stretching vibration of O—H. The signal at 2929.79 cm^{-1} indicated a C—H stretching vibration. The strong peak at 1626.40 cm^{-1} was caused by the stretching vibration of C═C. The strong peak at 1408.73 cm^{-1} represented the C—H vibration. The absorbances at 1250.52 cm^{-1} and 1074.18 cm^{-1} showed the C—O vibration in the acetyl group [19] and the existence of a pyranose ring, respectively. Furthermore, small peaks at 870.23 cm^{-1} and 835.10 cm^{-1} were attributed to the presence of mannose and α-type glucosidic bonds, respectively [20].

3.4.4. NMR analysis

NMR spectroscopy is the most powerful technique for detecting complex polysaccharide structures. It provides detailed structural information of carbohydrates, including the identification of monosaccharides, the elucidation of glycosidic bond types, and the establishment of linkage patterns [21].

The ^{1}H spectrum of AOFP1 is shown in Fig. 5D. The signals overlapped heavily due to hydroxyl shielding. In the anomeric proton region (4.50–5.50 ppm), AOFP1 contains seven anomeric proton signals at δ 4.57, 4.66, 5.05, 5.12, 5.16, 5.32 and 5.36, which are designated as seven sugar residues. The chemical shifts at 4.57 and 4.66 ppm were assigned to β-configuration monosaccharides, while the chemical shifts at 5.05, 5.12, 5.16, 5.32 and 5.36 ppm were assigned to α-configuration monosaccharides [22].

Fig. 5E depicts the ^{13}C NMR spectrum of AOFP1. Signals at 92–110 ppm (δ 92.41, 98.40, 100.03, 101.76, 103.18, 108.44, 109.26) in the ^{13}C spectrum indicated that AOFP1 had seven anomeric carbons. Additionally, δ 170.72 and 172.13 demonstrated that AOFP1 contained carboxyl carbons from Gal*p*A or Glc*p*A [23]. The δ_H/δ_C signal at 2.17/20.46 indicated the appearance of an acetyl group [24].

According to the HSQC NMR spectrum (Fig. 5F) and reported data [23–28], the signals at 4.57/103.18, 4.66/101.76, 5.05/100.03, 5.12/108.44, 5.16/109.26, 5.32/98.40 and 5.36/92.41 ppm were attributed to the C1/H1 of (1,4)-β-D-Gal*p*, (1,4)-β-D-Xyl*p*, (1,2,4)-α-D-Gal*p*A, (1,3,6)-α-D-Man*p*, (1,3,5)-α-L-Ara*f*, (1,4)-β-Glc*p*A, and (1,6)-3-acetyl-α-D-Glc*p*. Furthermore, the cross peaks observed at 3.52/80.12, 3.64/75.96, 3.89/76.73, 3.37/66.68 in the HSQC NMR spectrum indicated the C2/H2, C3/H3, C4/H4, C5/H5 signals of the (1,4)-β-D-Xyl*p* residue [29]. Similarly, the cross peaks at 81.07/4.30, 84.58/4.11, 83.91/4.24, 69.88/3.84 represented the C2/H2, C3/H3, C4/H4, C5/H5 signals of (1,3,5)-α-L-Ara*f* [27]. Additionally, the signals at 71.51 and 78.38 ppm were assigned to C-3 and C-4 of (1,4)-β-D-Gal*p* [25]. The signals at δ 70.20 and 72.60 were deemed to be the C4 and C5 of (1,6)-3-acetyl-α-D-Glc*p* [24].

4. Discussion

There are many kinds of southern traditional Chinese medicines in Hainan province, China. *Alpiniae oxyphyllae* fructus is one of the four famous southern traditional Chinese medicines. Here, the extraction, isolation, immunoregulatory activity, and characterization of AOFP were meticulously performed.

To obtain a higher yield of AOFP from *Alpiniae oxyphyllae* fructus, RSM was applied in the present work. RSM is an optimization method that combines experimental design and mathematical modeling [30]. This method obtains the optimal value for each factor after a multiple quadratic regression equation is used to fit the functional relationship between the factors and results in the whole range [31]. What is important is RSM has the advantages of higher precision and more accurate predictions compared to the traditional orthogonal experimental design method [32]. BBD is a design method in RSM that is suitable for experimental conditions with <5 factors and 3 levels and is used in many reaction optimization processes [18]. BBD is able to reduce the number of tests and effectively investigate the interaction between the influencing factors [33]. Additionally, BBD is widely used in the optimization of polysaccharide extraction from traditional Chinese medicine [34]. Therefore, the extraction conditions of AOFP were optimized using BBD in the present study. The optimal extraction conditions were a decoction temperature of 90 °C, a decoction duration of 60 min, a ratio of water to *Alpiniae oxyphyllae* fructus of 12:1 (mL:g), and an ethanol concentration of 75% (Table 3, Fig. 2). Moreover, the yield of AOFP from *Alpiniae oxyphyllae* fructus under such extraction conditions was 3.18%.

It is well known that polysaccharides extracted from traditional Chinese medicines are complex. Therefore, isolation to study AOFP is necessary. Recently, Han et al. [8] isolated polysaccharides in *Alpiniae oxyphyllae* fructus using a DEAE-52 cellulose column and a Sephadex G-100 column. However, in their elution with the DEAE-52 cellulose column, the linear elution of NaCl solution (tubes 21–80) started too early, before the elution of distilled water was complete (tubes 1–20) [8]. As a result, their first elution fraction was present between the NaCl eluent and distilled water eluent [8]. Additionally, they used too few elution tubes (80 tubes) so that the polysaccharides were eluted incompletely and only two fractions were obtained [8]. Therefore, isolation of the polysaccharides from *Alpiniae oxyphyllae* fructus was studied in the present work. We also isolated AOFP using a DEAE-52 cellulose column (50 tubes of distilled water and 120 tubes of NaCl solution) and a Sephadex G-100 column (100 tubes of distilled water) (Fig. 3). Three polysaccharides (AOFP1, AOFP2, and AOFP3) were eventually obtained.

Improving immunity effectively protects the body from viral diseases, cancers, parasitic infections, and other diseases [35,36]. Polysaccharides in traditional Chinese medicine often have immunoregulatory effects [10,37], which may be related to the fact that polysaccharides are one of the main substances recognized by the immune system [38]. Lymphocytes are immune cells that play important roles in the immune system through cellular immunity and humoral immunity. Lymphocyte proliferation is an important indicator that reflects the level of the immune response [39]. Many polysaccharides in traditional Chinese medicine stimulate lymphocyte proliferation. Selenylation of lily polysaccharides promoted lymphocyte proliferation [10]. Wusiman et al. [40] also reported that Alhagi honey polysaccharides enhanced lymphocyte proliferation. In the present study, we found that polysaccharides from *Alpiniae oxyphyllae* fructus possessed immunoregulatory activity via stimulation of lymphocyte proliferation (Fig. 4B and C). Notably, AOFP1 performed better than AOFP2 and

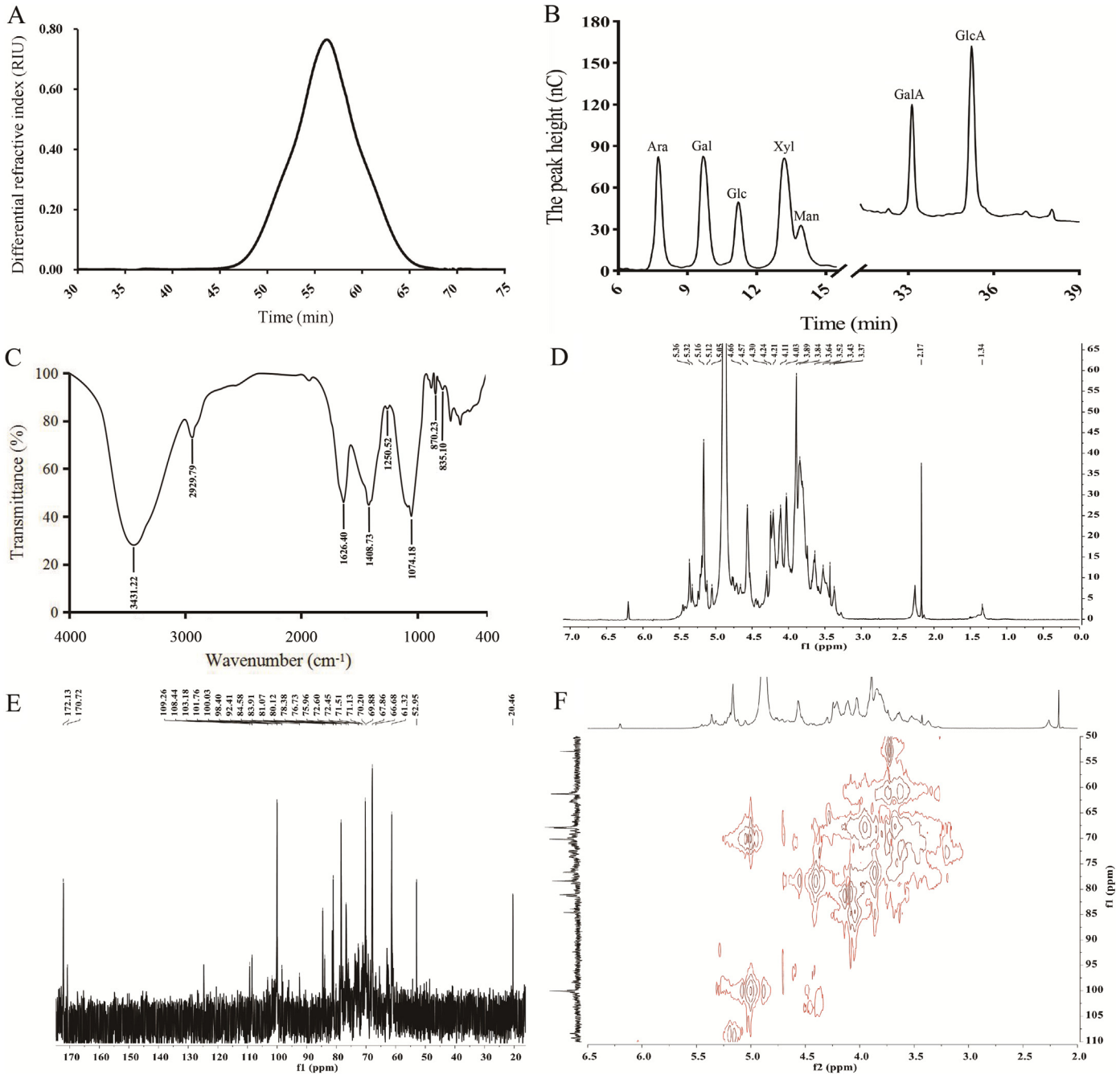

Fig. 5. Characterization of AOFP1. Molecular weight determination of AOFP1 was determined by GPC (A). Monosaccharide composition of the polysaccharide was determined by HPAEC-PAD (B). The IR spectrum (C) and NMR spectra (D, ^{1}H; E, ^{13}C; F, HSQC) were also used to characterize AOFP1.

AOFP3. Therefore, AOFP1 was the main study object in the next immunoregulatory effect and characterization analyses.

T lymphocytes contain auxiliary Th1 and Th2 cells, which are capable of producing Th1- and Th2-type immune responses. Th1 cells mainly secrete IL-2 and IFN-γ to mediate cellular immunity [41,42], and polysaccharides in traditional Chinese medicine often induce a Th1-type immune response. Sui et al. [43] concluded that *Radix Rehmanniae* polysaccharides significantly promoted the production of IL-2. Wusiman et al. [40] described that Alhagi honey polysaccharides accelerated IFN-γ secretion. When treated with AOFP1, IL-2 and IFN-γ levels significantly increased, which suggested that AOFP1 induced a Th1-type immune response (Fig. 4D and E). Th2 cells secrete IL-4 and IL-6 to mediate humoral immunity [43,44]. *Radix Rehmanniae* polysaccharides facilitate the production of IL-4 [43], and Alhagi honey polysaccharides accelerate IL-6 secretion [40]. In the present study, AOFP1 significantly stimulated IL-4 and IL-6 secretion (Fig. 4F and G). This result indicated that AOFP1 induced a Th2-type immune response. B lymphocytes also play an important role in specific immune responses. Most polysaccharides promote the production of specific antibodies by B lymphocytes [40,45]. We also found that AOFP1 promoted the production of OVA-sIgG (Fig. 4E). In summary, AOFP1 effectively enhanced the immune response, which was similar to the immune results of most studies on polysaccharides [3,46].

Here, the extraction, isolation, immunoregulatory activity, and characterization of polysaccharides from *Alpiniae oxyphyllae* fructus were comprehensively studied. We expect AOFP1 to be developed into a immunoregulatory drug in the future. Additionally, AOFP1, AOFP2, and AOFP3 are worthy of further study.

5. Conclusion

In summary, the optimal extraction conditions of AOFP were measured by BBD and extraction rate was greatly improved. Afterwards, the three polysaccharides (AOFP1, AOFP2, and AOFP3) were purified from crude AOFP by DEAE-52 cellulose and Sephadex G-100 columns. Then, AOFP1 not only enhanced Th1 and Th2 immune responses, but also effectively promoted specific immune responses. AOFP1 was composed of arabinose, galactose, glucose, xylose, mannose, galacturonic acid, and glucuronic acid at a molar ratio of 16.46:12.7:4.9:17.11:4.35:6.52:6, respectively. And the average molecular weight of AOFP1 was 43.4 kDa. What was more, combined with the anylasis of monosaccharide composition, FT-IR, and NMR spectra, the (1 → 4)-β-D-Xyl*p* and (1 → 3,5)-α-L-Ara*f* residues were mainly exist in the AOFP1.

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