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Optimization of extraction technology of the *Anemone raddeana* polysaccharides (ARP) by orthogonal test design and evaluation of its anti-tumor activity

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ABSTRACT

In order to get high quality bioactive polysaccharides (ARP) from *Anemone raddeana,* an orthogonal experiment ($L_9(3)^4$) was applied to optimize the best extraction conditions. Extraction time, extraction temperature, number of extraction and water to raw material ratio were the main factors to influence the yield and purity of the extracted crude polysaccharides. The four factors chosen for the present investigation were based on the results of a single-factor test. The optimum extraction conditions were determined as follows: extraction time 1.5 h, temperature 80 °C, number 4 and water to raw material at 6. Under optimized conditions, the experimental yield 7.97 ± 0.27% agreed closely with the predicted yield. In vivo ARP at suitable dose is effective on Th1 cells, as associated with an enhancement of IgG2a and IgG2b levels. In vitro ARP significantly inhibited the proliferation of human hepatocellular carcinoma HEP-G2 and human intestinal cancer HCT-22 cells, indicating ARP could be a potential anti-cancer therapeutic agent.

1. Introduction

Chinese traditional medicine declared the therapeutical effect of medicinal herbs for human disease might lie in the synergistic interaction of the many constituents. In such synergistic systems, activity results from the presence of multiple active principles that must be together to create the desired response (Pezzuto, 1997). When such complex mixtures are fractionated, the active ingredients are separated and efficacy may be lost. Thus the optimal extraction technology plays a key role in a wide variety of industries including cosmetics, pharmaceutical and food industries, etc.

"Liangtoujian", *Anemone raddeana* Regel, are widely distributed over the three provinces of northeast China, which is an Anemone herb belonging to the Ranunculaceae family. In ancient time, people usually drink the water-extractable physic liquor from the rhizome of *A. raddeana* as a drug for relieving rheumatism and subduing inflammation, anti-tumor and so on. Its indications are wind–cold–damp type of arthralgia, carbuncle swelling inflammation, spasm of limbs and scleromere ache (Wang, Cui, & Liu, 1985). Its main constituents include polysaccharides, saponins, internal ester, lipid and alkaloid (Lu, Xu, Zhang, & Sun, 2002; Zhou, Sun, Li, Wang, & Liu, 2007). The extensive modern pharmacological studies on this plant are mainly on its saponins portion. However, so far there is not any information published about the ARP extraction technology and its anti-tumor activity research. So in this study, we take orthogonal experiment to optimize the best ARP extraction conditions for getting high yield and quality bioactive polysaccharides.

2. Materials and methods

2.1. Materials

Anemone raddeana was purchased from a local shop (Changchun, Jilin Province, China).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from Sigma Chemical Co. Goat anti-mouse IgG2a and IgG2b peroxidase conjugate were from Southern Biotech. Medium Roswell Park Memorial Institute 1640 (RPMI-1640) was purchased from Gibco Invitrogen Co. The RMPI-1640 medium, used for anti-tumor activity tests, was supplemented with Hepes buffer 10 μmol/mL, penicillin 100 IU/mL, streptomycin 100 μg/mL, L-glutamine 2 μmol/mL, 2-mercaptoethanol 50 μmol/L and 10% newborn bovine serum, pH 7.2. Fetal calf serum (FCS) was provided by Hangzhou Sijiqing Corp. All other reagents were of grade AR.

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2.2. Experimental animals

Male ICR mice (Grade II, 5–6 weeks old) weighing 18–22 g, were purchased from the Pharmacology Experimental Center of Jilin University and acclimatized for 1 week prior to use. All mice were housed under standard conditions at 24 ± 1 °C, with humidity of 50 ± 10% and a 12/12 h light/dark cycle. Rodent laboratory chow pellets and tap water were supplied ad libitum. All the procedures conducted by the Institute for Experimental Animals of Jilin University were carried out in strict accordance with the PR China legislation on the use and care of laboratory animals and were approved by the university committee for animal experiments.

2.3. Extraction of ARP

The rhizome of *A. raddeana* (1000 g) was ground in a blender to obtain a fine powder (Particle diameter size: 400–500 μm) and then was extracted 3× with petroleum ether at 60 °C for 6 h to defat, and then 3× with 80% EtOH at 75 °C for 6 h to remove some colored materials, oligosaccharides and some small molecule materials under reflux in the apparatus, Soxhlet's. The pretreated samples were separated form the organic solvent by centrifugation (2000*g* for 10 min, at 4 °C). Each dried pretreated sample (20 g) was extracted by water in a designed time, temperature, number and water to raw material ratio. The water extraction solutions were separated form insoluble residue through the nylon cloth (Pore diameter: 38 μm), and then precipitated by the addition of ethanol to a final concentration of 75% (v/v). The precipitates collected by centrifugation (2000*g* for 10 min, at 20 °C) were solubilized in deionized water and lyophilized to get the crude polysaccharides. Total sugar content was determined by the phenol–sulfuric acid method using glucose as a standard (Sun et al., 2008). The percentage polysaccharides yield (%) is calculated as the polysaccharides content of extraction divided by dried pretreated sample weight (20 g).

2.4. Optimization of ARP extraction

An orthogonal $L_9(3)^4$ test design was used to investigate the optimal extraction condition of polysaccharides from *A. raddeana*. As seen from Table 1, the extraction experiment was carried out with 4 factors and 3 levels, namely extraction time (1.5, 2, 2.5 h), extraction temperature (80, 90, 100 °C), number of extraction (2–4) and water to raw material ratio (5–7). The range of each factor level was based on the results of preliminary experiments. The yield (%) of ARP was the dependent variable. The ARP obtained from the above 9 tests was operated following the method in the Section 2.3.

2.5. Immunization

Ovalbumin (OVA) was used model antigen. Male ICR mice were divided into five groups, each consisting of five mice. Animals were immunized subcutaneously on the back using OVA 100 μg alone or OVA 100 μg dissolved in saline containing ARP (200, 400 and 800 μg) on Day 1. Saline-treated animals were included as controls. Immunizations were performed twice at a 14-day interval and mice were sacrificed 14 days after the second immunization. Sera collected from animals on Day 28 were used for measurement of anti-OVA IgG2a and IgG2b by ELISA (Sun, Li, & Liu, 2008).

Table 1
Factors and levels for orthogonal test

Variable	Levels		
	1	2	3
(A) Extraction time (h)	1.5	2	2.5
(B) Extraction temperature (°C)	80	90	100
(C) Number of extraction (*n*)	2	3	4
(D) Ratio of water to raw material (*n*)	5	6	7

2.6. Measurement of OVA-specific antibody

OVA-specific IgG2a and IgG2b antibodies in serum were detected by ELISA according to the method previously described by Sjoelander et al. with some modifications (Sun & Liu, 2008). In brief, the wells of 96-well microtiter plates were coated with 100 μl of OVA solution (50 μg/ml in 50 mM carbonate buffer, pH 9.6) for 24 h at 4 °C. The wells were washed 3 times with 200 μl of PBS containing 0.05% (v/v) Tween 20 (PBS/Tween), and then blocked with 200 μl of 5% FCS/PBS at 37 °C for 1 h. After three washings with PBST, 100 μl of diluted serum samples (IgG2a, 1:100; IgG2b, 1:50) or 0.5% FCS/PBS as control was added to triplicate wells. The plates were then incubated for 1 h at 37 °C, followed by washing 3 times. Aliquots of 100 μl of horseradish peroxidase-conjugated goat anti-mouse IgG2a or IgG2b (diluted 1:4000 in FCS/PBS) were added and incubated for 1 h at 37 °C. After washing, 100 μl of TMB (3,3′,5,5′-tetramethylbenzidine) liquid substrate was added to each well. The plate was incubated for 15 min at 37 °C, and enzyme reaction was terminated by adding 50 μl/well of 2 N H_2SO_4. The absorbance was measured using the ELISA reader at 490 nm with a 595 nm reference. Data were expressed as the mean OD value of the samples minus the mean OD value of the control. Results were expressed as log2 titers. Where sets of serum samples have been subjected to within and between group comparisons, ELISA assays of all the samples were performed on the same day.

2.7. Cell proliferation assay

Human hepatocellular carcinoma HEP-G2 and human intestinal cancer HCT-22 cells were obtained from the Pharmacology Experimental Center of Jilin University. HEP-G2 and HCT-22cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 IU/ml) and streptomycin (100 μg/mL) (Cui et al., 2007; Hsu, Kuo, & Lin, 2004; Tomatsu, Ohnishi-Kameyama, & Shibamoto, 2003) under an atmosphere of 5% CO_2 at 37 °C. The proliferation of HEP-G2 and HCT-22 cells was determined using colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described previously (Cui et al., 2007). Briefly, an aliquot of 100 μL of cells (10^5/mL) was attached for 24 h in the 96-well plate, and then removed the supernatant. The polysaccharide (50, 100, 200, 400 μg/mL, final concentration) was seeded into each well for 48 h. The negative controls were treated with the medium only. About 10.0 μL of 0.4% MTT was added into each well and incubated for another 4 h. After the supernatant was aspirated, 100 μL Me_2SO was added to the culture and homogenized for at least 10 min to fully dissolve the colored material. The absorbance at 570 nm was measured on an ELISA reader (Model 680, Bio-RAD Instruments). The inhibition ratio of the treated cells was calculated based on the following formula: (1 − the A_{570} value for treated cells/the A_{570} value of untreated cells) × 100%.

3. Results and discussion

3.1. Effect of extraction time on extraction yield of ARP

The yield (%) of ARP affected by different extraction time (0.5–3.5 h) was seen in Fig. 1, when other three factors (extraction

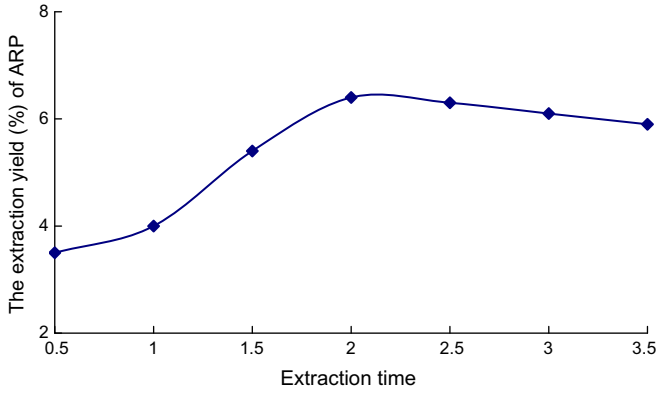

Fig. 1. Effect of different extraction time on the extraction yield of polysaccharides from *Anemone raddeana*.

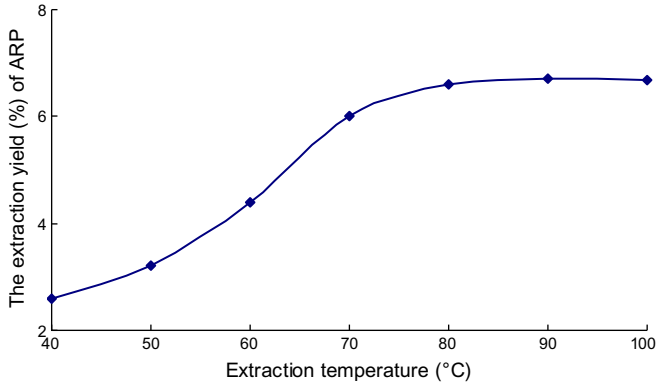

Fig. 2. Effect of different extraction temperature on the extraction yield of polysaccharides from *Anemone raddeana*.

temperature, number of extraction and water to raw material ratio) were fixed at 80 °C, 3 times and 6. The yield (%) of ARP increases with the increasing extraction time and reached the peak value (6.43 ± 0.13%) at 2 h and then dropped from 2 to 3.5 h.

3.2. Effect of extraction temperature on extraction yield of ARP

The yield (%) of ARP affected by different extraction temperature (40–100 °C) was seen in Fig. 2, when other three factors (extraction time, number of extraction and water to raw material ratio) were fixed at 2 h, 3 times and 6. The yield (%) of ARP increases with the increasing extraction temperature and reached the maximum value (6.68 ± 0.18%) when extraction temperature ranging from 80 to 100 °C.

3.3. Effect of number of extraction on extraction yield of ARP

The yield (%) of ARP affected by different number of extraction (1–7 times) was seen in Fig. 3, when other three factors (extraction time, temperature and water to raw material ratio) were fixed at 2 h, 80 °C and 6. The yield (%) of ARP gets the critical value (6.42 ± 0.22%) when the samples were extracted for 3 times. And then there is a little increase when extracted exceeds 3 times.

3.4. Effect of ratio of water to raw material on extraction yield of ARP

The yield (%) of ARP affected by different ratio of water to raw material (3–9) was seen in Fig. 4, when other three factors (extraction time, temperature and number of extraction) were fixed at 2 h, 80 °C and 3. The result implied the yield (%) of ARP was enhanced to the critical value (6.63 ± 0.31%) at the ratio of 6, and then it increased in a mild slope when the ratio of water to raw material increasing.

So in this study, we adopted extraction time of 1.5–2.5 h, extraction temperature of 80–100 °C, extraction number of 2–4 times and water to raw material ratio of 5–7 for further study objects in the orthogonal test design experiment.

3.5. Optimization of the extraction parameters of ARP

To the best of our knowledge, various parameters play a great role in the optimization of the experimental conditions for the development of a solvent extraction method. Extraction time, extraction temperature, number of extraction and water to raw material ratio are generally considered to be the most important factors that affect the yield (%) of ARP. The investigated levels of each factor were selected depending on the above experiment results of the single-factor. Independent variables with three variation levels, X1 (extraction time: 1.5, 2, 2.5 h), X2 (extraction temperature: 80, 90, 100 °C), X3 (number of extraction: 2–4) and X4 (water to raw material ratio: 5–7) are listed in Table 1. In the present study, all selected factors were examined using an orthogonal $L_9(3)^4$ test design. The total evaluation index was used to analysis by statistical method. The analysis results of orthogonal test, performed by statistical software SPSS 13.0, are presented in Table 2. The ARP obtained from each test was pretreated and quantitatively analyzed according to the method as above discussed in

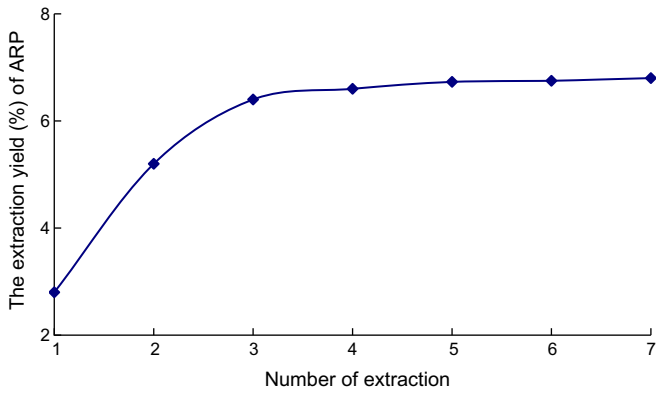

Fig. 3. Effect of different number of extraction on the extraction yield of polysaccharides from *Anemone raddeana*.

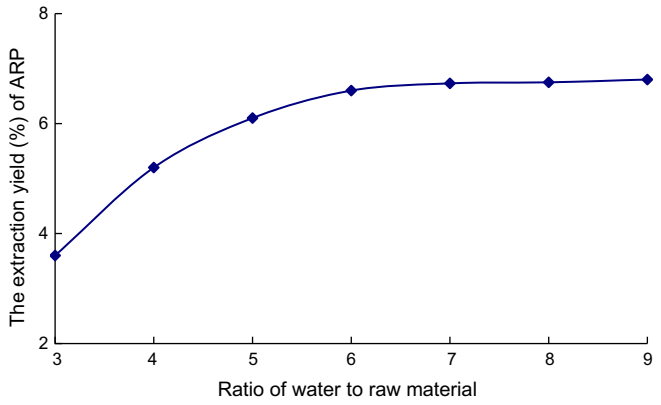

Fig. 4. Effect of different ratio of water to raw material on the extraction yield of polysaccharides from *Anemone raddeana*.

Table 2
Analysis of $L_9(3)^4$ test results

No.	(A) Extraction time (h)	(B) Extraction temperature (°C)	(C) Number of extraction (n)	(D) Ratio of water to raw material (n)	The yield (%) of ARP
1	1	1	1	1	5.67 ± 0.32
2	1	2	2	2	6.30 ± 0.21
3	1	3	3	3	7.25 ± 0.31
4	2	1	2	3	7.52 ± 0.45
5	2	2	3	1	7.18 ± 0.30
6	2	3	1	2	6.25 ± 0.14
7	3	1	3	2	6.56 ± 0.16
8	3	2	1	3	6.18 ± 0.22
9	3	3	2	1	6.89 ± 0.19
K_1	19.22	19.75	18.1	19.74	$\sum Y = 59.80$
K_2	20.95	19.66	20.71	19.11	
K_3	19.63	20.39	20.99	20.95	
R^a	1.73	0.73	2.89	1.84	

[a] Refers to the result of extreme analysis.

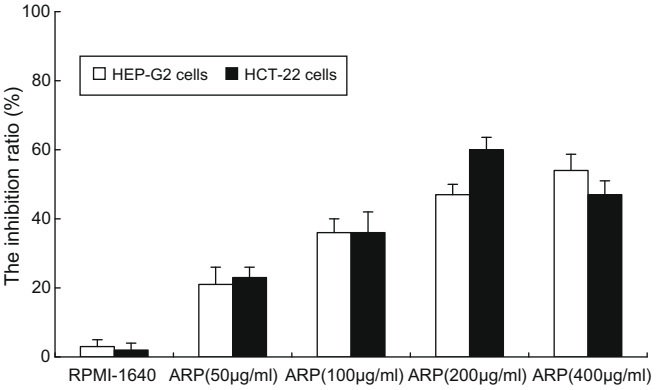

Fig. 6. The in vitro inhibition ratio of HEP-G2 and HCT-22 cells by the polysaccharides from *A. raddeana* at different concentrations.

Section 2.3. Although the maximum yield (%) of ARP was 7.52 ± 0.31%, we cannot choose the corresponding extraction conditions as the best technology. In view of orthogonal analysis, we adopt statistical software to calculate the values of K, k and R. The factors influence the yield (%) of ARP were listed in a decreasing order as follows: $C > D > A > B$ according to the R value. So the maximum yield of the polysaccharides was obtained when extraction time, extraction temperature, number of extraction and water to raw material ratio were $C_3\ D_3\ A_2\ B_3$ (4 times, 7, 2 h and 100 °C), respectively. According to the R value and the result of analysis of variance table (data not shown), we can find the number of extraction was found to be the most important determinant of the yield (%) of ARP. However, three levels of three other factors can be overlooked in the yield. In order to save the cost of production and time for industrialization, we make the optimum technology as follows: $C_3\ D_2\ A_1\ B_1$ (4 times, 6, 1.5 h and 80 °C). Through confirmatory test, we get the high yield and quality bioactive polysaccharides, with a yield (%) of 7.97 ± 0.27%.

3.6. Effect of ARP on the OVA-specific serum antibody response in vivo

The effect of ARP on the induction of humoral immune response in OVA immunized mice was evaluated. As shown in Fig. 5, ARP (200, 400 and 800 μg) significantly enhanced the OVA-specific serum IgG2a and IgG2b antibody levels, as compared with control group ($p < 0.01$), especially at a dose of 400 μg. Among the T lymphocytes, helper T cells induce B lymphocytes to secrete antibodies. Help T cells can be divided into two subsets of effector cells, namely Th1 and Th2 cells. The Th1 cells secrete cytokines, such as interleukin-2 (IL-2), tumor necrosis factor-β (TNF-β) and interferon-γ (IFN-γ), and stimulate the production of IgG2a, IgG2b and IgG3 in mice. They are responsible for cytotoxic T lymphocyte (CTL) production. The Th1 response against intracellular pathogens and malignant cells is superior to the Th2 response (Constant & Bottomly, 1997; Del Prete, De Carli, Ricci, & Romagnani, 1991; Fiorentino, Bond, & Mosmann, 1989). Thus, from the above findings we can draw a conclusion that ARP at suitable dose can enhance serum antibody production, and tilt the immune system in favor to Th1 type response in mice immunized with OVA.

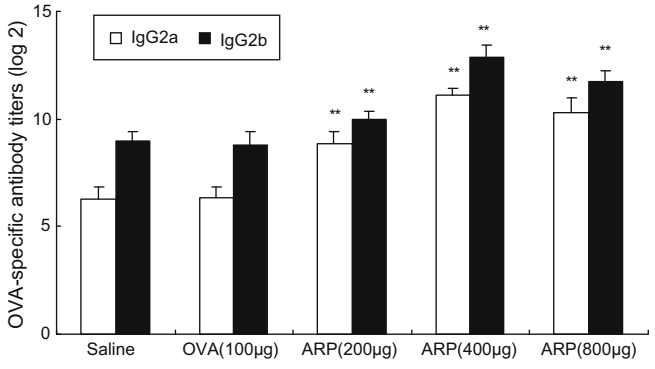

Fig. 5. Effect of ARP at different concentrations on OVA-specific IgG2a and IgG2b antibody. Significant differences with OVA group were evaluated using Student's t-test, $^{**}p < 0.01$.

3.7. Anti-tumor activity of ARP in vitro

In this study, the anti-tumor activity of ARP against two kinds of human solid cancer cell lines, HEP-G2 and HCT-22, was investigated in vitro. In Fig. 6, ARP significantly inhibited the proliferation of HEP-G2 cells ($p < 0.05$) at the concentrations ranging from 50 to 400 μg/ml. The tendency of anti-tumor effect was displayed in a concentration-dependent manner. At the maximum concentration of 400 μg/ml, ARP had the maximum inhibition ratio of 54.3 ± 4.7%. Meanwhile, the proliferation of HCT-22 was significantly suppressed at the concentration of 200 μg/ml, with the ratio of 60.2 ± 3.6%. The anti-tumor activity of the polysaccharide was usually believed to be a consequence of the stimulation of the cell-mediated immune response (Borchers, Keen, & Gershwin, 2004; Ooi & Liu, 2000; Sullivan, Smith, & Rowan, 2006). For instance, immunostimulatory activities were found in the polysaccharides from *Polyporus albicans*, *Grifola frondosa*, *Pleurotus ostreatus*, etc., which suggested that immunostimulatory effects might be the main mechanism for the anti-tumor activities of polysaccharides (Cui et al., 2007; Sarangi, Ghosh, Bhutia, Mallick, & Maiti, 2006; Sun et al., 2008). But some polysaccharides, such as polysaccharides from *Angelica sinensis* (Cao et al., 2006) and *Poria cocos* (Huang, Jin, Zhang, Cheung, & Kennedy, 2007), could directly inhibit the proliferation of cancer cell in vitro.

From above, we can draw a conclusion that ARP, obtained by the optimum extraction technology through orthogonal experiment methods, has enormous potential for use in medicine against both pathogens and cancer.

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