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RESEARCH ARTICLE

Study on separation and purification of oligomeric proanthocyanidin from *Rhodiola rosea*

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Abstract **The objective was to study the technology for the separation and purification of oligomeric proanthocyanidins from *Rhodiola rosea* and establish the best operating conditions. First, the oligomeric proanthocyanidins was extracted by ethyl acetate from rough-extracted *R. rosea* liquid using an optimized technique. Its purification was achieved by macroporous resins. Five kinds of macroporous adsorbent resins were compared for the adsorption and desorption performance of procyanidins, the concentration and pH value of both the extracted sample and the eluant were investigated. According to the results, the optimized conditions were as follows: four times of extraction at 25 min each time was effective, the best volume ratio was 1.5:1 (ethyl acetate: extracted solution); AB-8 resin was the best choice; the concentration of the extracted sample was 4.0 g/L, and the pH was 4.5; the ratio of the diameter to height of the chromatography column was 1:40 (cm); 50% ethanol was used as the eluant at pH 5; and finally, the purity of procyanidins reached 88. 3%.**

Keywords *Rhodiola rosea*, oligomeric proanthocyanidins, extraction, macroporous resins, separation and purification

Introduction

Rhodiola rosea, a genus of Crassulaceae, abundantly and wildly grows in the Xinjiang Uygur Autonomous Region and northern China. There is a variety of *R. rosea*, produced in the Zhangjiakou Region, Hebei Province. With its big root and high yield, it has a bright prospect for development. The introduction of *R. rosea* has already achieved success at present in the Chongli County, China. The root of artificially cultivated rhodiola can weigh 350 g, with yields reaching more than 2600 kg per acre (Wu et al., 1994). According to previous researches, *R. rosea* has a variety of health care functions such as enhancing human immunity (Farhath et al., 2005), antifatigue (Zhao et al., 2006), improving memory (Petkov and Yonkov, 1986) and antitumor, etc. Its usage as a new resource for food was approved by the Ministry of Health of the People's Republic of China in 1991, then it was added to the list of raw materials of health foods in March 2002.

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Procyanidins, the generic term of the flavan-3-ols derivatives, exist widely in plants and are polymerized by a different number of catechin or epicatechin monomers. Previous studies showed that procyanidins have numerous functions, including as a strong antioxidant and an ability to eliminate free radicals (Bagchi et al., 1997, Lu et al., 2004), prevent cardiovascular diseases (Takashi et al., 2005), and antitumor (Eng et al., 2003; Han et al., 2003) and antiaging abilities (Bagchi et al., 1998). Among the procyanidins, the oligomeric ones (dimer to tetramer) have aroused wide interest because of their high efficiency, low toxicity and high biologic utilization rates. Up to now, there are few reports about rose rhodiola procyanidins. Gad et al. (2006) showed that different varieties of *R. rosea* contained different polymerization degrees of procyanidins. Panossian et al. (2010) confirmed that *R. rosea* was rich in the procyanidins and that EGCG acted as their basic structural unit. According to Meng et al. (2007), there was 3.6% procyanidins in the *R. rosea* produced in the Zhangjiakou region, Hebei Province with the extraction rate of 5.43%. Compared with other plants, the content ranked only second to that of grape (*Vitis vinifera*) seed (7.88%) and sea buckthom (*Hippophae rhamnoides*) (8.14%), but was higher than that in *Pinus*

massoniana bark (2%), wild hawthorn fruit (*Crateagus pinnatifida*) (2.7%) and Japanese creeper (*Parthenocissus tricuspidata*) (1.407%)(Dong et al., 2010), etc. The extraction rate of 5.43% was higher than that in *Pinus massoniana* bark (5.12%), litchi (*Litchi chinensis*) (1.4%) and sea buckthorn (1.12%) (Wang et al., 2009; Zhou et al., 2009; Mei et al., 2010; Wen et al., 2010). Therefore, it is of great value for development. Until now, no researches about the separation and purification process of proanthocyanidins from *R. rosea* have been reported.

Common methods for the separation of procyanidins include organic solvent extraction, macroporous resin method, ultrafiltration, HPLC, glucan gel chromatography, etc. In this experiment, the remaining part of rhodiola after water extraction of polysaccharides was selected as the research material, ethyl acetate was used to extract the oligomeric proanthocyanidins from the *R. rosea*, and the macroporous adsorptive resin was utilized to accomplish the separation. In addition, the extraction separation was optimized in order to improve the reuse of *R. rosea*.

Materials and methods

Materials and instruments

R. rosea roots were collected in Zhangjiakou, Hebei Province; standard substances of procyanidins (⩾95%) were provided by Tianjin Jianfeng Natural Products Company. The UV-2802H type H uv-vis spectrophotometer was purchased from Unico (Shanghai) Instrument Co., Ltd. FD-1 type freezing dryer was provided by the Beijing Medical Equipment Factory. 95% ethanol, petroleum ether and ethyl acetate were analytically pure.

Methods of separation

The technological process was as follows: powdering of *R. rosea* roots →water extraction→supernatant→ethanol extraction→elementary extract of proanthocyanidins from *R. rosea*→washing with 75% ethanol→washing with petroleum ether→freeze-drying to obtain crude extracts of proanthocyanidins→reextraction by ethyl acetate to obtain crude extracts of oligomeric proanthocyanidins→ purification by macroporous absorption resin to obtain final purified extracts of oligomeric proanthocyanidins.

Detection of oligomeric proanthocyanidins from *R. rosea* was conducted using the hydrochloric acid-vanillin method (Meng et al., 2009).

The crude extracts of proanthocyanidins were prepared using 50% ethanol as the extracting solution at a material-to-solvent ratio of 1:25, followed by reflux-condensing for 2 h at 80°C with 3 replicates, merging and centrifuging of extracts to reserve the supernatant, and volatilizing ethanol to get the elementary extraction solution of proanthocyanidins. Removing sugar using four times the volume of 75% ethanol and fat with isopyknic petroleum ether, the extracts were concentrated and vacuum frozen-dried.

Oligomeric proanthocyanidin was extracted by ethyl acetate. In a single-factor experiment, the effects of extracting time, extraction times and the volume ratio were examined from the separation result of oligomeric proanthocyanidin from the crude extract of procyanidins. The combined effects were also examined in the orthogonal test choosing the L9 (33) orthogonal table; the analysis of variance was carried out using SPSS software in order to optimize the conditions of the extraction process. The level of factors are shown in Table 1.

Oligomeric proanthocyanidin was isolated and purified by macroporous absorption resin. First, the resin was immersed in 95% ethanol for 24 h, then washed with enough ethanol again, and finally, rewashed with distilled water until no alcohol flavor could be detected. Second, five kinds of accurately weighed macroporous resins (about 2.00 g) were put into several 150 mL sealed flasks with 80 mL of the crude oligomeric proanthocyanidin solution at certain concentrations, then shaken under 120 r/min at (25±1)°C for 24 h and filtered well to determine the concentration of the proanthocyanidin residue in the filtrate. The proanthocyanidin residue was washed with deionized water and 50 mL 95% ethanol, respectively, then shaken at 120 r/min at (25±1)°C for 24 h to ensure desorption. Finally, the concentration of proanthocyanidin residue in the filtrate was detected, with the adsorbance and desorption rate of each kind of resin calculated.

Through the resin static adsorption of the procyanidin experiment, the concentration and pH value of both the sample liquid and desorption solution, and the diameter-to-height ratio of the chromatography column were investigated and optimized by dynamic adsorption and desorption tests based on the single factor experiment. The factor and levels of the orthogonal test are shown in Table 2; the variance was analyzed by SPSS software.

Table 1 Factors and levels of orthogonal test

Levels	Factors		
	A (extracting time)	B (volume ratio)	C (extraction times)
1	25	1.5:1	2
2	30	2:1	3
3	35	2.5:1	4

Table 2 Factors and levels of orthogonal test

Levels	Factors		
	A (pH of extract)	B (pH of strippant)	C (diameter-to-height ratio)
1	3.5	4.0	1:40
2	4.0	5.0	1:25
3	4.5	6.0	1:15

Determination of proanthocyanidin content

Determination of the content of oligomeric proanthocyanidins in the product was conducted by the vanillin-hydrochloric acid method.

Results and analysis

Catechin and its oligomer have the same weak polarity as ethyl acetate, while water is a kind of polar solvent with hydrogen bonding. According to the theory that solutions with similar polarity are more mutually soluble, the "solvent vacancy" formed in ethyl acetate needs less energy than in water; consequently, oligomeric procyanidin is mostly dissolved in the ethyl acetate phase while high polymer procyanidin is mainly dissolved in the water phase.

As shown in Fig. 1, the rate of extraction increased along with the time, reached its peak (92%) after 30 min and thereafter decreased. Therefore, 30 min was supposed to be suitable for this extraction process.

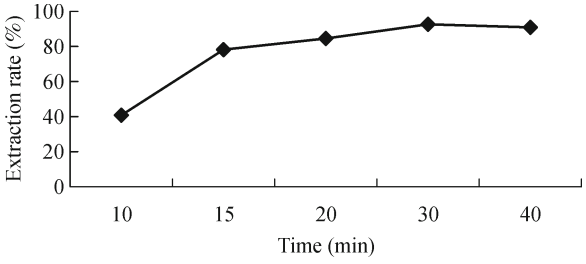

Figure 1 Effect of extraction time on the extraction rate.

Figure 2 reveals that when the ratio of ethyl acetate and crude procyanidins was 0.5∶1, the extraction rate was less than 80%. The extraction ratio significantly increased along with the increase of ethyl acetate; however, when the volume ratio became 2∶1, the extraction rate tended to be stable. Consequently, the volume ratio of 2∶1 (ethyl acetate: the crude procyanidin) was selected, comprehensively considering the consumption of reagents, environmental protection and other factors.

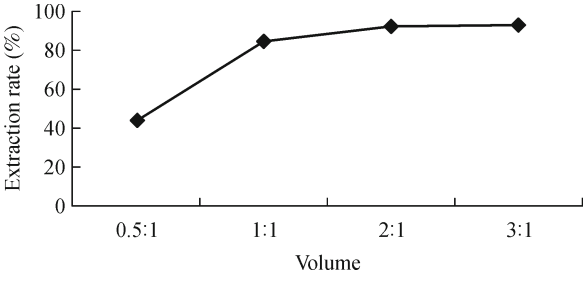

Figure 2 Effect of volume of ethyl acetate on the extraction rate.

From Fig. 3, it can be seen that the extraction rate had an upward trend along with the increase of extraction times, reaching 93% after 3 times of extraction, and then remaining stable. Therefore, three times of extraction is supposed to be economical, comprehensively considering the consumption of reagents, environmental protection and other factors.

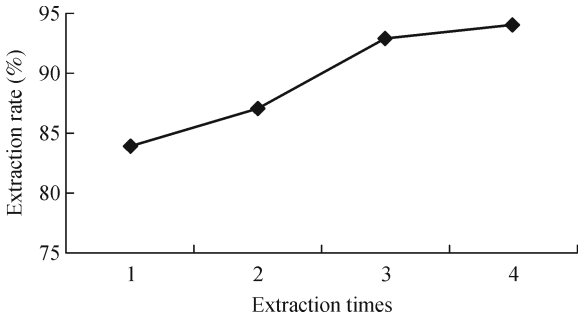

Figure 3 Effect of extraction times on the extraction rate.

According to the results in Table 3, the optimized conditions for the extraction are extraction for 30 min at the volume ratio of 1.5∶1(ethyl acetate: the crude procyanidins) with 4 replicates of operation. The factor influences on the test were ranked as follows: the extraction times > the volume ratio > the extracting time. And from Table 4 we could find out that the effect of extracting time was not significant, therefore, 25 minutes of extraction was chosen to save time. As a conclusion, the best choice of conditions for the ethyl acetate extraction were the extracting time of 25 minutes, the volume ratio of 1.5∶1(ethyl acetate: the crude extraction of procyanidins) and 4 times of extraction.

As is shown in Table 5, all of the selected resins have certain degrees of oligomeric proanthocyanidin adsorbability, however AB-8 macroporous resin exhibits the highest adsorption, and it also has priority judging from the desorption rate. Therefore, AB-8 macroporous adsorption resin was determined as the most suitable resin for the purification of oligomeric proanthocyanidin from *Rhodiola rosea*.

The highest absorbance was acquired when the concentration of the stock solution was 4.0 mg/mL (Fig. 4). A neither too low nor too high concentration was beneficial to the adsorption of oligomeric proanthocyanidin on the chromatography column. When the concentration was too low, part of the resins failed to reach the saturated state, while leakage would reduce the resin efficiency and cause a longer time to reach saturation; however, when the concentration was too high, the leak point would appear earlier, also leading to the appearance of flocculation and precipitation, which could easily cause pollution and congestion to the resins, reducing their adsorption ability. Therefore, 4.0 mg/mL was chosen as the concentration of the stock solution on adsorption in this experiment.

As shown in Fig. 5, the adsorption efficiency of pH AB-8

Table 3 Results of orthogonal test on extraction

Test numbers	Factors				
	A	B	C	Content (mg/mL)	
1	1	1	1	1.98	1.98
2	1	2	2	2.45	2.59
3	1	3	3	2.27	2.17
4	2	1	2	2.33	2.33
5	2	2	3	2.35	2.57
6	2	3	1	1.96	2.14
7	3	1	3	2.85	2.65
8	3	2	1	1.88	1.70
9	3	3	2	2.23	2.19
K1	13.49	14.12	11.63		
K2	13.67	13.54	14.12		
K3	13.49	12.95	14.86		
k1	2.25	2.35	1.94		
k2	2.28	2.26	2.35		
k3	2.25	2.16	2.48		
R	0.03	0.19	0.54		

Table 4 The variance analysis of orthogonal experiment

Sources of variation	Sum of square deviations	Degree of freedom	Mean square	*F* value	Critical value *F*	Significance
Extraction time	0.005	2	0.003	0.254	$F_{0.01}(2,9) = 8.02$	non-significant
Volume ratio	0.112	2	0.056	5.473		significant
Extraction times	0.948	2	0.474	46.275	$F_{0.05}(2,9) = 4.26$	very significant
Error	0.092	2				

Table 5 The structure characters of resins and their proanthocyanidins adsorption properties

Resin types	Polarity	Appearance	Particle size (mm)	Specific surface area (m^2/g)	Average pore size (nm)	Adsorbance (mg/g)	Desorption rate (%)
S-8	polar	yellowish	0.30–1.25	100–120	28.0–30.0	53.5	49.0
HPD750	medium	white	0.30–1.20	650–700	8.5–9.0	51.1	66.6
AB-8	weak	oyster white	0.30–1.25	480–520	13.0–14.0	55.5	70.9
HPD100	nonpolar	white	0.30–1.20	650–700	8.5–9.0	54.2	68.4
D101	nonpolar	white	0.30–1.25	480–550	10.0–11.0	51.2	66.9

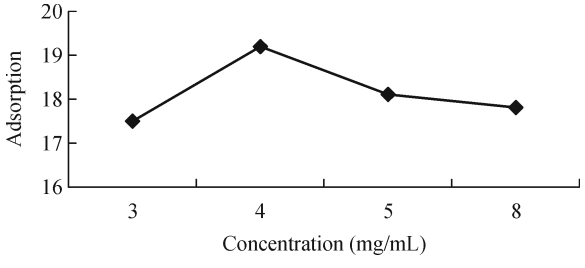

Figure 4 Effect of the concentration of the stock solution on adsorption.

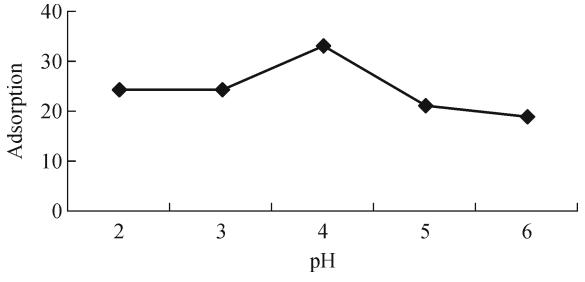

Figure 5 Effect of pH of the stock solution on adsorption.

macroporous resin was relatively higher when the pH value of the stock solution on adsorption was 4.0. Because the pH value had an effect on the degree of dissociation of the active principle and the affinity between the effective components

and the solvent, it influenced the degree of difficulty of the effective component adsorption by the macroporous adsorption resin. Because of the large amounts of phenolic hydroxyl with weak acidity in the molecule, procyanidins showed better stability and adsorption efficiency under acidic conditions.

In Fig. 6, the desorption rate of oligomeric proanthocyanidin increases along with the increase of ethanol concentration at the beginning, then at more than 50% ethanol concentration, the desorption rate is stabilized, with no significant changes. Consequently, 50% ethanol solution was chosen as the eluant.

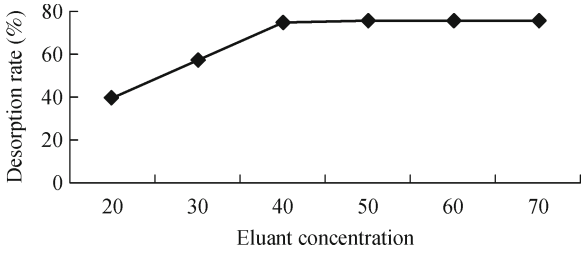

Figure 6 Effect of eluant concentrations on the desorption rate.

The elution rate of oligomeric proanthocyanidin increased with the rise of pH value at first, reached the highest effect at the pH value of 5, but then decreased with the approach to a neutral pH of 6 (Fig. 7). The acidic medium could destroy the combination of procyanidin and proteins, polysaccharides, or cellulose, so as to improve the elution rate of procyanidin and prevent the oxidation of procyanidin in the elution process. Thus, pH 5.0 was the best choice.

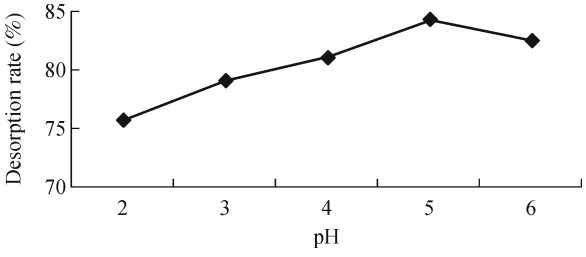

Figure 7 Effect of pH of eluant on desorption rate.

As for the optimization of conditions, an orthogonal test choosing the L9 (33) orthogonal table was adopted, considering 3 relatively better levels of the pH of stock solution, the pH of the eluant and the diameter-to-height ratio of the chromatography column. The results of the range analysis are shown in Table 6, with the variance analyzed by the SPSS software as shown in Table 7.

According to Table 6 and Table 7, the optimum conditions for the macroporous resin purification process was A3B2C1, namely, the pH value of the stock solution was 4.5, the pH value of the eluant was 5, and the diameter-to-height ratio was 1:40. The factor influences on the test was the pH value of the stock solution > the pH value of the eluant > the diameter-to-height ratio. The influences of the pH value of both the stock solution and the eluant were significant, while the effect of the diameter-to-height ratio on the purification showed no significant difference.

Table 6 Result of orthogonal test on macroreticular resins

Test numbers	Factors			
	A	B	C	Content (mg/mL)
1	1	1	1	1.31
2	1	2	2	1.17
3	1	3	3	0.96
4	2	1	2	1.35
5	2	2	3	1.54
6	2	3	1	1.11
7	3	1	3	1.43
8	3	2	1	1.53
9	3	3	2	1.42
K1	3.44	4.09	3.95	
K2	4.00	4.24	3.94	
K3	4.38	3.49	3.93	
k1	1.15	1.36	1.32	
k2	1.33	1.42	1.31	
k3	1.46	1.16	1.31	
R	0.31	0.26	0.01	

Conclusions

The optimal conditions for the extraction of oligomeric proanthocyanidins from *R. rosea* by ethyl acetate were: extraction for 25 min with 4 replicates at the volume ratio of 1.5:1(ethyl acetate: the crude procyanidins).

AB-8 macroporous adsorptive resin proved to be most suitable for separating oligomeric proanthocyanidins from *R. rosea* compared with other materials.

The concentration of the stock solution at 4 mg/mL and the pH value of 4.5 were supposed to be reasonable, with 50%

Table 7 The variance analysis of the orthogonal experiment

Sources of variation	Sum of square of deviations	Degree of freedom	Mean square	*F* value	Critical value *F*	Significance
pH value of the stock solution	0.688	2	0.344	9.135	$F_{0.01}(2,9) = 8.02$	very significant
pH value of the eluant	0.570	2	0.285	7.566		significant
Diameter-height ratio	0.096	2	0.048	1.276	$F_{0.05}(2,9) = 4.26$	non-significant
Error	0.688	9	0.038			

ethanol solution as the desorption solution at pH 5 and the diameter-to-height ratio of 1:40 of the chromatography column.

The purity of the separated products was 88.3% when measured by the vanillin-hydrochloric acid method.

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