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Ultrasonic-assisted extraction of flavonoids from *panax notoginseng* flowers and its antimicrobial activities

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ABSTRACT

Ultrasonic-assisted extraction of flavonoids from Panax notoginseng flowers (PNF) and its antimicrobial activities were first investigated. Single-factor and orthogonal experiment were used to investigate the optimum extraction condition. The results showed that, the combination of 90% ethanol, material-to-liquid ratio of 1:20 (g/mL), 1 h ultrasonic extraction and 3 times of extraction were optimal extraction condition with the highest yields of flavonoids from PNF at 40 kHz/200 W. Under the optimal extraction condition, the extraction rate was 2.49%. Also, flavonoids from PNF had obvious inhibitory effects on Staphylococcus aureus, Aeromonas hydrophila and Pseudomonas aeruginosa. These results clearly indicate that flavonoids from PNF have the potential to be antimicrobial agents, and also provide the theoretical data for supporting the use of PNF for food, pharmaceutical, cosmetics or nutraceutical industries.

Keywords: *Panax notoginseng* flowers, Ultrasonic extraction, Purification, Antimicrobial activity.

INTRODUCTION

Panax notoginseng is a well-known traditional Chinese medicinal herb and its flowers also have pharmaceutical efficacy including clearing heat, calming the liver, treating hypertension, vertigo, tinnitus, etc. The amount of total saponins in the flowers of *P. notoginseng* (PNF) is higher than in any other parts of *P. notoginseng*, in addition, the PNF also contains rich flavonoids and polysaccharide of bioactivity constituents [1-3].

Flavonoids have significant biological activities, such as antioxidant, anti-cancer and anti-microbial, etc [4-7]. Up to now, only few reports on flavonoids from PNF have been found: Huang et al. isolated and identified two flavanoids from buds of *P. notoginseng* [8] and Wang et al [9] investigated the optimum extraction condition of flavonoids from PNF by using orthogonal experimental design. Many studies on PNF mainly focused on content determination, structure identification and total pharmacological efficacy of saponins as well as various substances identification of volatile oil while relatively lacking in extraction and structural identification of flavonoids as well as its antibacterial activity [10-12].

Ultrasonic-assisted extraction has been applied extensively in food and medical industries in recent years due to its advantages of simple operation, short extraction time, good reproducibility and relatively high efficiency, etc. However, there haven't been any reports on flavonoids extracted from PNF by ultrasonic-assisted method or report on its antibacterial activity. In this study, ultrasonic-assisted extraction and orthogonal experimental design were used to optimize extraction flavonoids from PNF for the first time. Also, the antimicrobial activities of purified flavonoids on several bacteria were evaluated by using agar plate diffusion method.

EXPERIMENTAL SECTION

2.1. Materials and chemicals

PNF were provided by Takada Wenshan Panax Notoginseng Co., Ltd., (Yunnan, China). PNF were dried at 60°C in an oven and ground to obtain fine powder (60 mesh). Ascorbic acid, ABTS and rutin (95% purity) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and reagents in present study were analytical grade. AB-8 and ADS-7 were purchased from the Chemical Plant of Nankai University (Tianjin, China).

2.2. Extraction of flavonoids and optimization by orthogonal array

1 g PNF powder was extracted in KQ5200DB numerical controlled ultrasonic cleaner (Kun Shan Ultrasonic Instruments Co., Ltd., Jiangsu Province, China) with a certain volume solvent under the selected frequency and power combination of 40 kHz/200 W and then the extracts were filtrated and diluted to the appropriate proportion.

The diluted solution was used to determine the contents of flavonoids. The effects of ethanol concentration (40, 50, 60, 70, 80, 90 and 100%), material ratio (1:5, 1:10, 1:15, 1:20, 1:25, and 1:30), ultrasonic extraction time (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 h) and extraction number (1, 2, 3 times) on extraction efficiency of total flavonoids were investigated. Finally, orthogonal experimental design was used to select optimal extraction condition after single factor experiments. In order to assure the accuracy of the experimental data, the experiment was performed in triplicate.

2.3. Determination of flavonoid content

The content of flavonoids was determined using a colormetric method described by Zheng et al [13] with some modifications. Briefly, the extraction solution and different concentrations of the rutin standards solution were diluted appropriately and mixed with $NaNO_2$ (1 mL, 5%, w/v). After 5 min, 1 mL $AlNO_3$ (10%, w/v) was added and allowed to stand for 6 min, then NaOH (10 mL, 4%, w/v) was added to the mixture. The mixture was adjusted to 25 ml with 30% ethanol. Measurement of absorbance was taken at 510 nm after 15 min. The content of flavonoids in extracting solution was determined according to rutin calibration curve ($Y=0.4695X-0.0100$, $R^2= 0.9991$). The experiment was performed in triplicate.

2.4. Purification of flavonoids by macroporous resin adsorption

The crude flavonoids-enriched extract obtained under the optimized condition was purified using a column (25 ×1.5 cm^2) packed with AB-8 macroporous adsorption resin according to the reference [14]. The conditions for purifying the flavonoids by AB-8 resin were: injecting concentration 3.75 mg/mL, pH = 5, injecting velocity 2.0 mL/min, 40% (v/v) ethanol as desorption solvent, desorption velocity of flow 1.5 mL/min. The purified extract of flavonoids was collected and evaporated at 50 °C, and was then freeze-dried for determination of antimicrobial activity.

2.5. Antibacterial activity assay

The bacteriostatic circles diameter of the extraction flavonoids on some bacteria was determined according to the previous report[15]. Bacteria (*Staphylococcus aureus* ATCC25922, *Bacillus megaterium* CICC 0.217, *Pseudomonas aeruginosa* CCTCC2620 and *Escherichia coli* ATCC25922) were cultured in the LB medium at 37°C, while *Aeromonas hydrophila* XS91-4-1 culturing in the TSA medium at 28°C, and the cultures were diluted by corresponding medium to the appropriate cell density. The diluted cultures (100 μL) were added to the LB or TSA agar at 60°C and the mixtures (cell density for 10^7organism /mL) were poured into a flat plate. The LB or TSA agar with bacteria were holed at diameter of 0.6 cm, and then the different concentration of flavonoids (80 uL) were added to relevant holes. The negative control was 30% ethanol, and the positive control was Levofloxacin Hydrochloride. After incubating at 37°C or 28°C for 24 h, bacteriostatic circles of flavonoids against several bacteria were observed and photographed.

RESULTS AND DISCUSSION

3.1. Single factor experiment

3.1.1. Effects of ethanol concentration on extraction efficiencies of flavonoids

The combination of material-to-liquid ratio of 1:20, 1 h soaking time, 1 h ultrasonic extraction time and once extraction was performed at 40 kHz/200 W ultrasonic cleaner. The impact of ethanol concentration of 40%, 50%, 60%, 70%, 80%, 90%, and 100% on the extraction yield of total flavonoids was analyzed. The result showed that ethanol concentration had an impact on extraction efficiencies of flavonoids from PNF. When ethanol concentration was 40-90%, with the increase of ethanol concentration, extraction yield of flavonoids gradually increased. Among the above seven kinds of ethanol concentrations, 90% concentration of ethanol produced the highest extraction yield of flavonoid, which had no significant difference with extraction yield from 80% ethanol (Figure 1-a). The results

were different from those of the reports of Wang et al. [9] on flavonoids extracted from PNF. Yet whether 90% concentration of ethanol is the optimum extraction solvent still needs further research.

3.1.2. Effects of material ratio on extraction efficiencies of flavonoids

With increase of material ratio (1:5, 1:10, 1:15, 1:20), the extraction efficiencies of flavonoids from PNF rose under certain conditions. When the material ratio was over 1:20, the extraction yield of flavonoids began to reduce (Figure 1-b). Probably because the material ratio (1: 20) had almost fully dissolved the flavonoids, other impurities can also be dissolved when the material liquid ratio increased again, which possibly affected the extraction yield of flavonoids. Considering both of the cost and efficiency of extraction, 1:20 was an appropriate ratio.

3.1.3. Effect of ultrasonic extraction time on extraction efficiencies of flavonoids

Effect of extraction time on extraction yield of flavonoids was similar with that of material ratio. The extraction efficiency of flavonoids also increased with the prolonging of extraction time (0.5-1.5 h) (Figure 1-c). However, the extraction efficiency began to decrease slowly with the prolonging of extraction time (more than 1.5 h), which may be due to the fact that after long time of ultrasonic treatment, solution temperature rose gradually, which influenced the stability of the flavonoids and led to the decrease of the extraction yield. Therefore, 1.5 h is an appropriate extraction time for the extraction of flavonoids.

3.1.4. Effect of extraction times on extraction efficiencies of flavonoids

Considering the cost of solvent and extraction difficulty, PNF was successively extracted once, twice and three times in this experiment. The Figure 1-d showed that with the increase of extraction times, the yield of total flavonoids gradually increased.

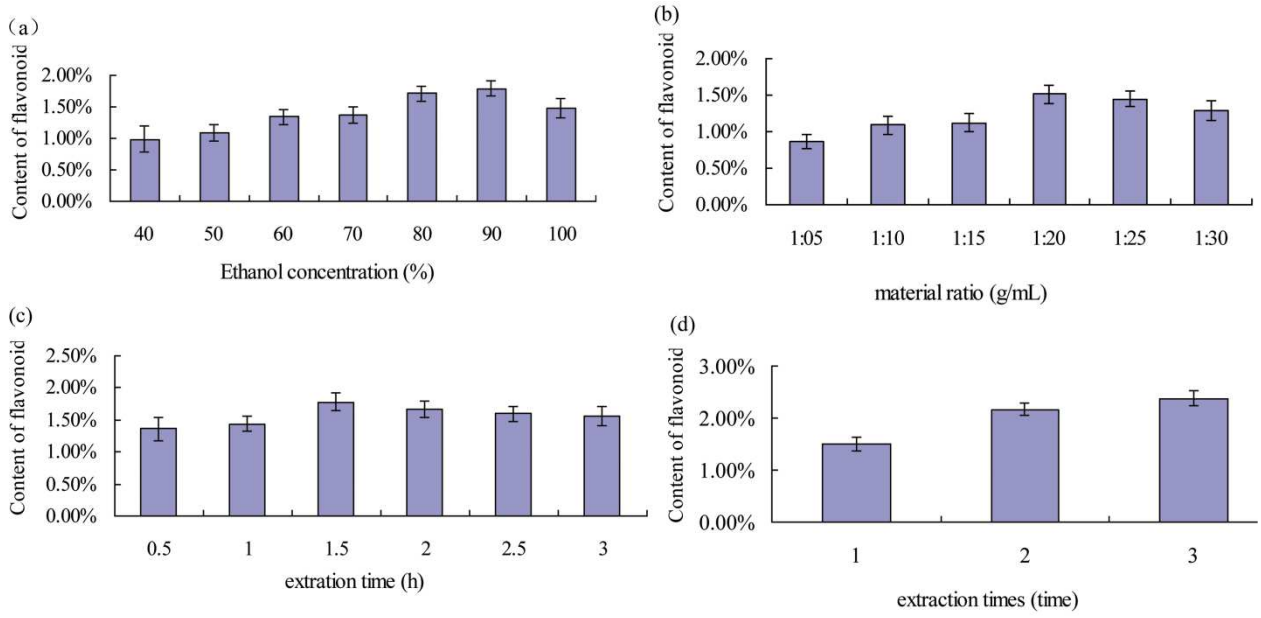

Figure 1. Effects of different extraction condition on extraction efficiencies of flavonoids. Ethanol concentration(a), material ratio (b), extraction time (c), extraction times (d)

3.2. Optimization of ultrasonic extraction condition of flavonoids from PNF

The influence of single factor on extraction efficiency of flavonoids from PNF was discussed in the former chapters. In order to optimize the ultrasonic extraction condition, orthogonal experimental design was introduced with four factors and three levels on the basis of single factor experiment (Table 1). Different process conditions led to certain differences in the total yield of flavonoids as shown in Table 2. As seen from Table 2, we found that the influence factors to the extraction efficiency of flavonoids from PNF decreases in the order: D > B> A > C according to the R values. That is, the main factors which influenced the extraction efficiency of flavonoids, in turn, were extraction times > material ratio> ethanol concentration > ultrasonic extraction time. Extraction times were found to be the most important determinant of extraction yield of PNF. As shown in Table 3, extraction times and material ratio were both significant influence on the extraction yield of flavonoids from PNF according to the variance analysis of the experimental factor. The best combination was A3B2C1D3 for flavonoids from PNF. Therefore, A3B2C1D3 (90% ethanol concentration, material ratio of 1:20, 1 h ultrasonic extraction and three successive extraction times) combination was finally selected as the optimum ultrasonic extraction condition of flavonoids in PNF on the basis of single-factor test. Under the optimum condition, the extraction yield of flavonoids from PNF was 2.49 %, which was

1.17 times higher than the highest yield of the previous report (2.146%) without ultrasonic-assisted extraction [9]. Hence, the results show that ultrasonic assisted extraction can improve the extraction yield of flavonoids from PNF.

Table 1 Factors and levels in $L_9(3^4)$ orthogonal experimental design

Factor	Ethanol concentration	Material ratio	Ultrasonic extraction time	extraction times
	A (%)	B（g/mL）	C (h)	D (times)
Level Ⅰ	70	1：15	1	1
Level Ⅱ	80	1：20	1.5	2
Level Ⅲ	90	1：25	2	3

Table 2 Orthogonal experimental results

Test number	Factor				Result (%)
	A	B	C	D	Flavonoid
1	1 (70%)	1 (1：15)	1 (1 h)	1 (1 time)	1.20
2	1	2 (1：20)	2 (1.5 h)	2 (twice)	1.75
3	1	3 (1：30)	3 (2 h)	3 (three times)	2.36
4	2 (80%)	1	2	3	1.94
5	2	2	3	1	1.53
6	2	3	1	2	2.15
7	3 (90%)	1	3	2	1.69
8	3	2	1	3	2.49
9	3	3	2	1	1.86
K1	1.77	1.61	1.95	1.53	
K2	1.87	1.92	1.85	1.86	
K3	2.01	2.12	1.86	2.26	
R	0.24	0.51	0.10	0.73	

Table 3 Variance analysis of the experimental factor

Variation source	Sum of squares	Degree of freedom	F-ration	Fcritical value	Significance Pvalue≤0.05
A	0.089	2	5.235	19.000	
B	0.402	2	23.647	19.000	*
C	0.017	2	1.000	19.000	
D	0.809	2	47.588	19.000	*
Error	0.02	2			
Total variation	1.317	10			

In order to further verify the result of orthogonal experiment method, five parallel experiments were performed under the optimum conditions. The average extraction yield was 2.49% ± 0.898% (Mean ± RSD), which showed that the experimental design was reasonable with good reproducibility and process stability.

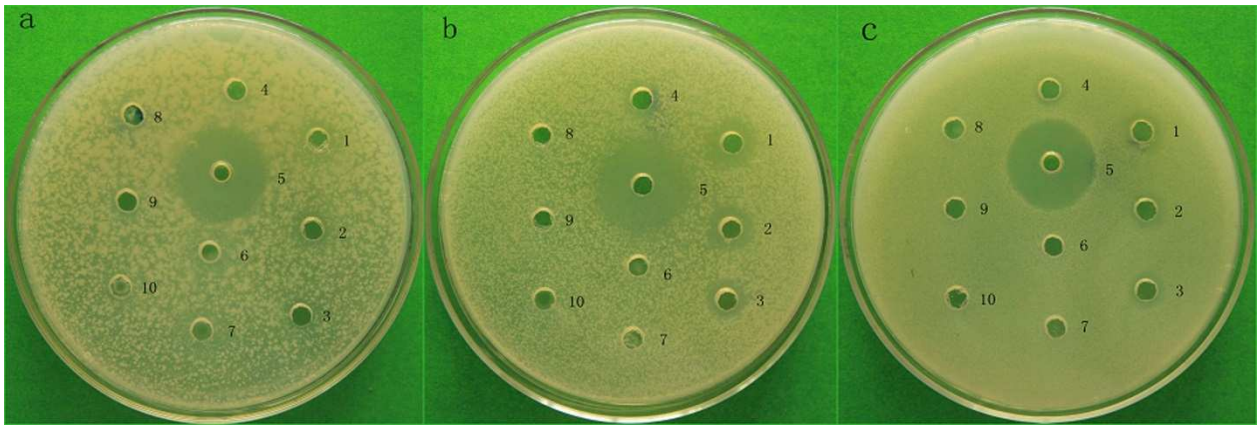

Figure 2. The antibacterial activity of the flavonoids from PNF on some bacteria

***Note: a**. Pseudomonas aeruginosa CCTCC2620; **b**. Staphylococcus aureusATCC25922; **c**. Aeromonas hydrophila XS91-4-1; 1-4: the different concentrations of flavonoid (369, 184.5, 92.25, 46.125 μg/mL); 5-8: Levofloxacin Hydrochloride as the positive control with different concentrations (10, 0.78, 0.39, 0.195 μg/mL); 9-10: the 30% ethylalcohol as the negative control.*

3.3. Antibacterial activity assay

The antibacterial activities of flavonoids from PNF against bacteria were shown in Figure 2. Bacteriostatic circles

indicated that the flavonoids had significant inhibitory effects on *Aeromonas hydrophila* and *Staphylococcus aureus* at more than or equal to 46.125μg/mL concentration as well as *Pseudomonas aeruginosa* at more than or equal to 184.5 μg/mL concentration. The antibacterial activities assay indicated that flavonoids from PNF had different inhibitory effect on different bacteria. This may be related to the types of flavonoids from PNF, but the specific kinds of flavonoids and their contents need to be further explored.

CONCLUSION

The combination of 90% ethanol concentration, material ratio of 1:20, 1 h ultrasonic extraction and three successive extraction times was proved to be the optimum ultrasonic extraction condition of flavonoids in PNF under 40 kHz/200 W according to the results of single-factor and orthogonal experiments. Under the optimum extraction condition, the content of flavonoids was 2.49%. The results also indicated that ultrasound-assisted extraction was an effective method for the extraction of flavonoids from PNF. In this study, flavonoids from PNF showed a broad spectrum of antimicrobial activity on Gram-positive and negative bacteria. And it had different inhibitory effect on different bacteria. The present study clearly indicates that flavonoids from PNF have the potential to be antimicrobial agents.

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