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Extraction of bio-active components from *Rhodiola sachalinensis* under ultrahigh hydrostatic pressure

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Abstract

A new extraction technique, ultrahigh hydrostatic pressure (UHP), was used to obtain bio-active components from *Rhodiola sachalinensis*. The leaching-out rates of flavones and salidroside were measured under different conditions. The extraction efficiency of different extraction methods and the DPPH radical scavenging activities of their crude extracts were investigated. The optimal conditions of extracting flavones and salidroside were as follows: 41 or 60% (v/v) of ethanol concentration, 70:1 ml/g of solvent–herb ratio, and 500 MPa of hydrostatic pressure for 3 min. The leaching-out rate of flavones and salidroside was up to 5.233 and 0.411%, respectively. The yield of crude extract of UHP was up to 26.116%, comparing with the crude extract was 13.825% by reflux and 16.995% by soxhlet. The UHP extraction technique showed high efficiency in extracting bio-active components at room temperature. The crude extract had stronger DPPH radical scavenging activity than TBHQ.
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Keywords: *Rhodiola sachalinensis*; Ultrahigh hydrostatic pressure; Room temperature; Salidroside; Flavones

1. Introduction

Rhodiola is a traditional Tibetan medicine in China with adaptogenic properties. It is mainly used as brain tonic and roborant for alleviating headache. It has a pronounced antifatigue effect reflected in an antifatigue index [1]. Current researches indicate that the extracts of Rhodiola could activate the synthesis or resynthesis of ATP in mitochondria, stimulate reparative energy processes after intense exercise [2], exhibit an antiinflammatory effect and protect muscle tissue during exercise [3], reduce the degree of hyperplasia in the erythropoietic stem under conditions of conflict situation [4], protect the liver from repetitive injury induced by carbon tetrachloride in rats [5], and increase NO synthesis in interferon-primed macrophages by providing a second triggering signal for the synergistic induction of iNOS mRNA expression [6]. The major bio-active components in Rhodiola species are phenylethanol derivatives (rhodioloside or salidioside, tyrosol), flavones (quercetin, hyperoside, kaempferol, rhodiolin, rhodionin, tricin, acetylrodalgin, catechins and proanthocyanins), phenylpropanoids (rosavin, rosin and rosarin) and the others including phenolic acids, ethyl gallate, ethereal oil, organic acids and lipids [7,8]. The components with high pharmacological values are salidioside and flavones.

The first step of analysis and utilizing the medicinal plant bio-active constituents is extraction, that is, the separation of components to be analyzed or used from the cellular matrix. The “ideal” extraction method should be quantitative, nondestructive, and timesaving. There are various methods for extracting salidroside such as leaching-out extraction [9], reflux extraction [10–12], ultrasonic extraction [13], and soxhlet extraction [14,15]. Microwave extraction is the only one method reported for flavones extraction [16,17]. Besides long extracting time, most of these methods have to employ heating which could easily lead to some thermo-sensitive ingredients losing their biological activities or transforming into other substances. The ultrahigh hydrostatic pressure (UHP) technique, as identified by US FDA, which ranges from 100 to 800 MPa, has been widely used in ceramics, graphite, casting industry, pharmaceutics, metallurgy, plastic making, civil engineering and food industry [18]. The UHP was first used to be an extraction technique by Zhang et al. in 2004 [19], and had exhibited excellent advantages in natural product extraction field. Studies showed that UHP technique could shorten processing time (within 10 min), obtain higher extraction yield, and had no negative effect on the activity and structure of bio-active components. Above all, this extraction technique could be operated at room

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temperature. It had been successfully used for extraction of icariin from *Epimedium*, polyphenols from green tea [19], flavones from *Propolis* [20], and ginsenoside from *Panax quinquefolium* [21].

We employed the UHP technique to extract flavones and salidroside from *Rhodiola sachalinensis* (*R. sa.*) to serve as a substitute for soxhlet extraction, reflux extraction, and other methods. The DPPH radical scavenging activity of crude extracts was determined to measure the antioxidant activity in vitro.

2. Methods

2.1. Plant material and reagents

R. sa. was cultivated in Yanji, Jilin Province of China. Lutin, as the standard samples, was purchased from the National Institute for the Control of Pharmaceutical and Biological Products, China. Salidroside, as the standard samples, was purchased from Beijing Xiantong Age Medicine Science Ltd., China. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), was purchased from Sigma–Aldrich company, Germany. Tertiary butylhydroquinone (TBHQ), was purchased from Fluka company, Denmark. Methanol was of high performance liquid chromatography (HPLC) grade and purchased from Fisher Scientific company, USA. All solutions were filtered through a 0.45 μm hydrophilic polypropylene membrane before HPLC analysis.

2.2. Apparatus

DL700 high hydrostatic pressure instrument (Da Long Machine Factory, Shanghai, China) was used for the UHP treatment. UV-2500 ultraviolet–visible (UV–vis) spectrophotometer (Shimadzu, Japan) was used to measure the content of flavones. LC-6A HPLC system (Shimadzu, Japan) was used to measure the content of salidroside which comprises of following parts: SCL-20A system controller, LC-20AB pumps, SIL-20A autosampling injector, CTO-20AC column oven, SPD-20A UV–vis photodiode array detector, and C-R3A data processor.

2.3. Ultrahigh hydrostatic pressure extraction procedure

The roots of *R. sa.* were dried in vacuum at 60 °C for 24 h, then pulverized and sieved. Powders of *R. sa.* through 40 mesh screen were mixed with a given amount of solvents, after being sealed, the mixture was subjected to UHP treatment at given pressure for a given time, then the pressure was quickly released. The extraction solution was centrifuged at a speed of 4000 rpm for 10 min, and the supernatant was collected and stored at 4 °C in refrigerator for further analysis.

2.4. Crude extracts preparation

The supernatant sample was evaporated by a rotary evaporator under vacuum at 60 °C, and then it was lyophilized in freeze-dryer. The yield of extracts was calculated as the weight (g) of crude extracts obtained from 100 g raw materials, expressing as percentage.

2.5. Quantitative analysis methods

2.5.1. Evaluation of the leaching-out rate of flavones (LRF) values

According to the method in literature [17], rutin was selected as the standard sample, the content of flavones was measured by colorimetry. The regression equation was $M = 0.750A + 0.000$ ($r^2 = 0.9996$, M was expressed as mg, and A represented the absorbance at 510 nm). The leaching-out rate of flavones was calculated as the weight (g) of flavones in the extracting solutions obtained from 100 g raw materials, expressing as percentage.

2.5.2. Evaluation of the leaching-out rate of salidroside (LRS) values

According to the method in Pharmacopoeia of China (2005) [22], the content of salidroside was measured by HPLC. The analyses were performed with a Shim-pack ODS column (150 mm × 4.6 mm I.D., 5 μm) at a column temperature of 25 °C. The mobile phase composed of methanol and ultrafiltration water (15:85) was eluted at a flow-rate of 1.0 ml/min and the effluent monitored at 275 nm. The injection volume was 5 μl and the mass of samples was determined by the external standard curve of salidroside. The regression equation was $M = 0.2851P(\mathrm{E}{-5}) + 0.0375$ [$r^2 = 0.9999$, M was expressed as μg, and P was the peak area of salidroside at retention time (12.376 min)]. The leaching-out rate of salidroside was calculated as the weight (g) of salidroside in the extracting solutions obtained from 100 g raw materials, expressing as percentage.

2.5.3. Evaluation of the DPPH radical scavenging activity of crude extracts

According to the method in literature [23] with slight modifications. The 0.2 mM solution of DPPH radical in ethanol was prepared and 2 ml of this solution was added to 2 ml of 0.5 mg/ml water solution containing different crude extracts. After 30 min at 25 °C, the absorbance was measured at 517 nm. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The scavenging activity of DPPH radical in percentage was calculated by the following equation: Scavenging activity (%) $= (1 - A_1/A_0) \times 100\%$, where A_0 was the absorbance of the control reaction and A_1 was the absorbance in the presence of crude extracts and TBHQ.

2.6. Statistical analysis

Each experimental data are the average value of three samples. An analysis of variance using the Student F test was performed to determine differences between means of the treatments at $p \leq 0.05$.

Table 1
Effect of solvent on leaching-out rates of constituents

Extracting solvent	LRF (%)	LRS (%)
Water	1.631	0.101
70% ethanol solution	3.949	0.326

Conditions: solvent–herb ratio was 30:1 (ml/g), hydrostatic pressure was 300 MPa, pressure holding time was 1 min.

3. Results and discussion

3.1. Determination of UHP extraction method

3.1.1. Effect of extracting solvent

The effect of extracting solvent was investigated for two solvents, i.e., water and 70% (v/v) ethanol solution. Flavones and salidroside were polar compounds that had polyhydroxyl groups, they could be easily dissolved in water and alcohol solution. Due to the variety of polarities of extraction solvents, the solubility of bio-active components and the rate of mass transfer are different. As shown in Table 1, the ethanol solution was more suitable to obtain higher yields of flavones and salidroside.

3.1.2. Effect of hydrostatic pressure

The effect of hydrostatic pressure was investigated in the range of 100–500 MPa. Table 2 indicated that the leaching-out rates of flavones and salidroside were all evidently increased when the hydrostatic pressure increased. Obviously, the UHP technique is useful for improving the leaching-out of flavones and salidroside. According to Le Chatelier's theory [24], the volume of system tends to reduce during the pressure promoting period. In this process, the extracting solvent comes into cells to integrate with bio-active components. Besides, the pressurized cells show increased permeability. The higher the hydrostatic pressure is, the more solvent can enter cells and the more compounds can permeate out to the solvent. The equilibrium of solvent concentration between inner and outer of cells would be established during the pressure holding period. When the ultrahigh pressure is suddenly released the cell wall is disrupted to release the cytoplasm which contains a high concentration of target material, so a short extraction time is enough to harvest high concentration of extract. Under ultrahigh pressure, bigger molecules (protein, starch, and so on) were denaturated and they would not come into the solvent, so the extent of impurities of UHP extract is less than other extraction methods. Furthermore, in the extraction process with high pressure, the solubility of extracts is improved as the pressure increases, so the leaching-out rates of bio-active components are improved.

Table 2
Effect of hydrostatic pressure on leaching-out rates of constituents

Treatment pressure (MPa)	LRF (%)	LRS (%)
100	3.780	0.223
200	3.825	0.246
300	3.949	0.326
400	3.994	0.334
500	4.089	0.340

Conditions: extracting solvent was 70% ethanol solution, solvent–herb ratio was 30:1 (ml/g), pressure holding time was 1 min.

Table 3
Effect of solvent–herb ratio on leaching-out rates of constituents

Solvent–herb ratio (ml/g)	LRF (%)	LRS (%)
10:1	3.503	0.263
20:1	3.709	0.281
30:1	3.949	0.326
40:1	3.960	0.331
50:1	4.322	0.334
60:1	4.424	0.353
70:1	4.436	0.362

Conditions: extracting solvent was 70% ethanol solution, hydrostatic pressure was 300 MPa, pressure holding time was 1 min.

3.1.3. Effect of pressure holding time

The effect of pressure holding time was investigated in the range of 1–7 min. Based on above analysis, the main function of pressure holding time is to form the equilibrium of solvent concentration between inner and outer of cells and to get in full touch with bio-active components and solvent. The different pressure between inner and outer cell membrane is so large that it will lead to instant permeation. Table 3 showed that the leaching-out rates of flavones and salidroside had no significant increase when the pressure holding time was beyond 3 min. Therefore, 3 min was enough to complete the equilibrium. According to Pascal theory [24], during the UHP treatment process, the pressure could transfer to the whole material uniformly and instantaneously. So the rate of pressure transfer rapidly with no stress gradients, which make the extraction process easy and effective, with heating unnecessary.

3.1.4. Effect of solvent–herb ratio

The effect of solvent–herb ratio was investigated in the range of 10:1–70:1 ml/g. Table 4 showed that the leaching-out rates of flavones and salidroside increased along with the accretion of ethanol solution amount. The dissolve process of boi-active components into the solvent was a physical process. When the amount of solvent increased, the chance of bio-active components coming into contact with extracting solvent expanded, leading to higher leaching-out rates. Taking into account of cost, the solvent–herb ratio should be limited to below 70:1.

3.1.5. Effect of ethanol concentration

The effect of ethanol concentration was investigated in the range of 30–90%. Table 5 indicated that its effects on flavones

Table 4
Effect of pressure holding time on leaching-out rates of constituents

Pressure holding time (min)	LRF (%)	LRS (%)
1	3.949	0.326
3	4.106	0.334
5	4.095	0.332
7	4.118	0.329

Conditions: extracting solvent was 70% ethanol solution, solvent–herb ratio was 30:1 (ml/g), hydrostatic pressure was 300 MPa.

Table 5
Effect of ethanol concentration on leaching-out rates of constituents

Ethanol concentration (%)	LRF (%)	LRS (%)
30	4.826	0.206
50	4.871	0.313
70	3.949	0.326
90	1.558	0.331

Conditions: solvent–herb ratio was 30:1 (ml/g), hydrostatic pressure was 300 MPa, pressure holding time was 1 min.

Table 7
Results of variance analysis

Target	Variance	Square sum	Freedom degree	F
	SR	68.982	8	
LRF	SE	0.016	1	530.130
	ST	68.999	9	
	SR	0.058	5	
LRS	SE	0.000	4	415.371
	ST	0.0584	9	

and salidroside were quite different. The leaching-out rate of flavones increased with the increasing of ethanol concentration till up to 50%, and decreased beyond 50%. The reason may be related to solvent polarity and the solubility of flavones. However, the leaching-out rate of salidroside became increasing when the ethanol concentrations increased. When the ethanol concentration was beyond 70%, the increasing extent slowed down. Taking into account of cost and the extraction of flavones, moderate concentration ethanol solution should be chosen as extracting solvent.

3.1.6. Determination of optimal process

Based on the single factor experimental results above, aside the request of instrument and cost, we chose the hydrostatic pressure (100–500 MPa), ethanol concentration (20–60%), solvent–herb ratio (30:1–70:1) as factors for optimization. The leaching-out rates of flavones and salidroside were determined. The pressure holding time of each sample was 3 min. Even Design's Software V4.0 was used to plan the test and analyze the results. The test design and results are shown in Table 6, the analysis is shown in Table 7.

The optimum regression equation of flavones leaching-out is as follows: $\mathrm{LRF} = 23.109 + 0.044A + 0.746B + 403.556C - 1.390AC - 1.477BC$ ($r^2 = 0.9998$, $F = 530.130 > F_{0.05}(8,1) = 239$, where A is the hydrostatic pressure, B is the ethanol concentration, and C is the solvent–herb ratio).

The most significant factor influencing the leaching-out rate of flavones was solvent concentration, the second was solvent–herb ratio, and the last was hydrostatic pressure. The optimal extraction efficiency of flavones could take place when the ethanol concentration is 41%, the solvent–herb ratio is 70:1, and the hydrostatic pressure is 500 MPa. The predicted value is 5.330%, and the value of proof-test is 5.233%. When the herb was extracted twice, the leaching-out rate of salidroside reached 6.178%.

The regression equation of salidroside extraction process is $\mathrm{LRS} = 0.010 - 0.0001A + 0.007B + 0.0005C - 0.00003BC$ ($r^2 = 0.9998$, $F = 451.371 > F_{0.05}(5,4) = 9.01$, where A is the hydrostatic pressure, B is the ethanol concentration, and C is the solvent–herb ratio).

The most significant factor influencing the leaching-out rate of salidroside is solvent–herb ratio, the second is solvent concentration, and the last is hydrostatic pressure. The optimal extraction efficiency of salidroside could take place when the ethanol concentration is 60%, solvent–herb ratio is 70:1 ml/g, and hydrostatic pressure is 500 MPa. The predicted value is 0.418%, and the value of proof-test is 0.411%. When the herb was extracted twice, the leaching-out rate of salidroside reached 0.425%.

3.2. Extraction efficiency of different extraction methods

Because of species and provenances diversity, the contents of flavonoids and salidroside in Rhodiola may be different. The raw material used in this research must be different from that given in the literature. In order to compare the effect of UHP with other traditional extraction methods, we performed related experiments, and the extraction methods were exactly followed what given in the literature. They were ultrasonic extraction [22], leaching extraction [9], soxhlet extraction [14], and reflux extraction [11].

Because leaching extraction empolys water as the extracting solvent, it was difficult to leaching-out flavones and salidroside. In ultrasonic extraction, reflux extraction, and soxhlet extraction methanol were utilized as the extracting solvent, since high concentration methanol has the same extracting effect as high

Table 6
Homogeneous design and experiment results

No.	Hydrostatic pressure (MPa)	Ethanol concentration (%)	Solvent–herb ratio	LRF (%)	LRS (%)
1	200	60	70:1	4.253	0.370
2	400	20	70:1	4.725	0.207
3	500	30	40:1	4.770	0.224
4	400	60	30:1	4.286	0.385
5	300	40	50:1	4.913	0.280
6	200	20	30:1	4.601	0.146
7	500	50	60:1	5.085	0.358
8	300	40	50:1	4.931	0.277
9	100	30	60:1	4.500	0.206
10	100	50	40:1	4.515	0.317

concentration ethanol, also hinders leaching-out of flavones. Soxhlet extraction brings the sample to contact with fresh solvent repeatedly, thus could obtain the whole salidorside. If the extraction yield of soxhlet was calculated as 100%, the salidorside yield of UHP extraction is 84.74%.

Table 8 showed that the UHP extraction for 3min gave higher leaching-out rates of flavonoids and salidroside than the other extractions for several hours, and the extraction efficiency was the highest one. Thus, UHP extraction can greatly shorten the extraction time. Limited by the energy level, weak bonds, such as the hydrogen bond, the electrostatic bond, the Van der Waals bond and the hydrophobic bond, could be broken by high pressure but the covalent bond could not be broken, which meant the small molecules would not change under UHP [18]. It was assured that the structures of flavones and salidroside would not change during the UHP process. Fig. 1 showed the HPLC curves of salidroside standard, UHP extract, and soxhlet extract. Fig. 2 showed the curve of UHP extraction solution after purified by D101 resin, the retention time of salidroside was changed from 12.376 to 13.161 min because we added an advanced-column to the analysis column. That is, the peak 1 with retention time 13.106 min in Fig. 2(b) was the exact one with retention time 12.375 min in Fig. 1(b). Then we will find the peak with retention time 16.214 min in Fig. 1(b) was diminished after treatment with D101 resin. The result showed that the compound might be some protein, polysaccharide or something else that we regard as impurities. Thus, we know that the salidorside and main peaks of UHP extract and soxhlet extract were the same, and the extent of impurities of UHP extract is less than other extraction methods.

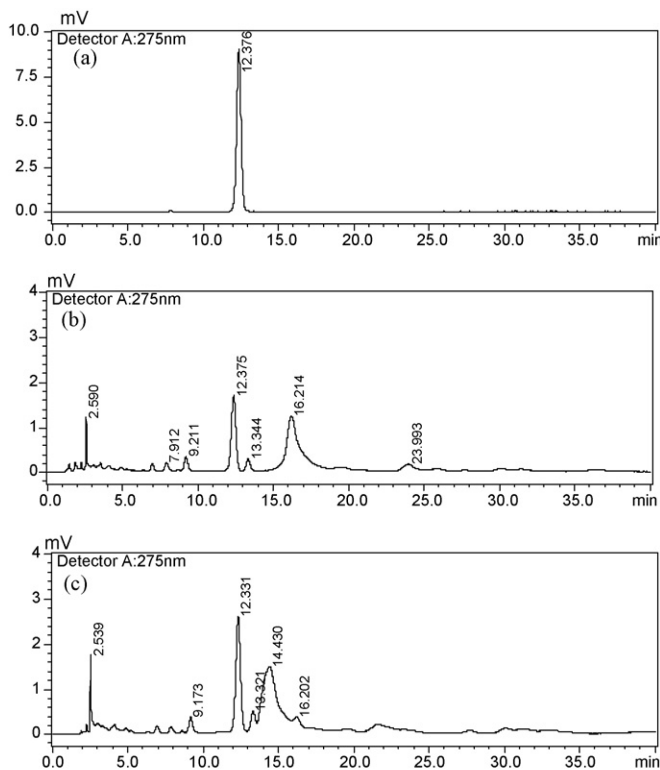

Fig. 1. Extraction efficiencies of different extracting methods. (a) HPLC curve of salidroside standard solution, (b) HPLC curve of UHP extraction solution, and (c) HPLC curve of soxhelt extraction solution.

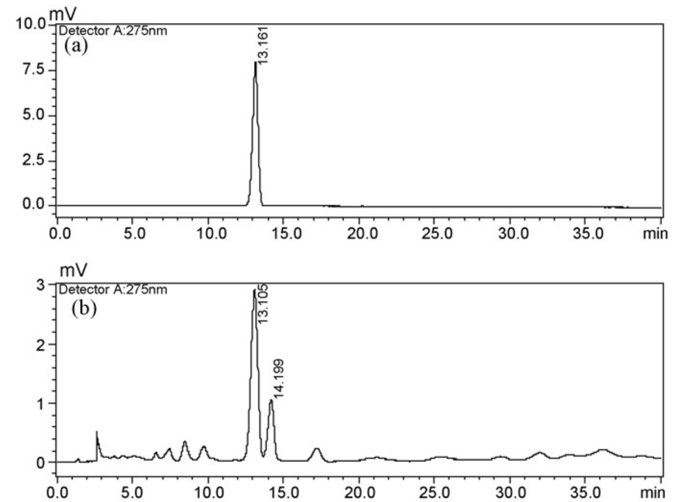

Fig. 2. Effect of D101 resin. (a) HPLC curve of salidroside standard solution; (b) HPLC curve of UHP extraction solution. Attention: the retention time of salidroside was changed from 12.376 to 13.161 min after adding an advanced-column to the analysis column.

3.3. DDPH radical scavenging activity of crude extracts

Although there are some synthetic antioxidant compounds, such as TBHQ, which are commonly used in processed foods, it has been reported that these compounds have some side-effects. Therefore, many researchers have focused on natural antioxiant sources. The flavones and polyphenolic substances are all provided with antioxidant properties. The composition of crude extracts obtained would be different due to different extration method. Fig. 3 showed the DPPH radical scavenging activities of crude extracts obtained by different extraction methods, they were higher than that of TBHQ, especially ultrasonic extract and soxhlet extract. Though the DPPH radical scavenging activity of UHP extraction was not the highest, due to the highest yield of crude extract, the UHP extraction had obviously superiority to obtain high quantity of antioxidant substances.

4. Conclusion

UHP extraction conditions of flavonoids and salidroside from *R. sa.* have been studied. UHP extraction can use moderate concentration of ethanol at room temperature to achieve

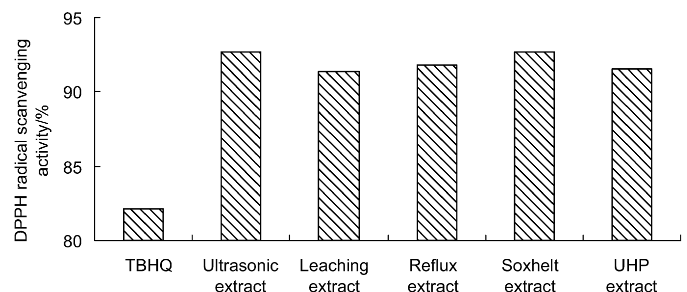

Fig. 3. DPPH radical scavenging activities of crude extracts.

Table 8
Extraction effects of different extracting methods

Extraction method	Extracting solvent	Extracting conditions	LRF (%)	LRS (%)	Yield of crude extract (%)
Ultrasonic extraction	Methanol	15:1, 40 Hz, 150 W, 30 min	2.050	0.288	14.420
Reflux extraction	Methanol	4:1, 80 °C, 120 min	1.519	0.302	13.825
Leaching extraction	Water	20:1, 100 °C, 80 min	1.969	0.315	15.522
Soxhlet extraction	Methanol	80 °C, 240 min	2.100	0.485	16.995
UHP extraction	41%, 60% ethanol	70:1, 500 MPa, 3 min	5.233	0.411	26.116

high extraction efficiency of flavones and salidroside in short time. Compared with those traditional extraction methods, UHP extraction provided much more flavones and higher extraction yield of crude extract which has higher DPPH radical scavenging activity than TBHQ.

The crude extract should be subjected to further separation and purification. The composition and the pharmacological properties of flavones need to be further studied. Furthermore, the UHP technique could be used combined with other techniques such as ultrasonic extraction and enzymatic extraction to improve the extracting rate and extracting efficiency.

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