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Compositional analysis and antioxidant activity assessment of flavonoids extracted from *Trollius chinensis* Bunge

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ABSTRACT

Flavonoids from Trollius chinensis Bunge.(Flos Trollii) were extracted by different method were investigated in the present study. Simultaneous determination of two flavonoids(orientin and vitexin) was accomplished by a useful, rapid and simple HPLC/DAD-ESI-MS method. A central composite design (CCD) combined with response surface methodology(RSM) was used to study the effects of extraction time, temperature and liquid to solid ratio on the extraction yield of vitexin and orientin from Trollius chinensis Bunge. The optimum extraction conditions found by maximizing the dependent variables were time of 70min, temperature of 48 ℃and liquid to solid ratio of 16g/mL, where 1.87% yield was predicted. The results are valuable for further utilizing and development Trollius chinensis Bunge. as a medicinal plant.

Keywords: *Trollius chinensis* Bunge.; HPLC/DAD-ESI/MS; Flavonoids; Antioxidant activity

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INTRODUCTION

Trollius chinensis Bunge.(Flos Trollii) is a perennial herb in Ranunculaceae's family[1], which includes about 30 species connected with temperate and arctic regions of the northern hemisphere[2]. In china, it's a medicinal plant which flower has already been included in the "Chinese Pharmacopoeia". Trollioside[3], flavonoids[4-6], trolliamide[7], phenolic acids, fatty acids[8-9], alkaloids[10], terpenoids[11], steroidsa[12]and other compounds were isolated from Flos Trollii in previous phytochemical research. The flower of Flos Trollii own antiviral[4,5], antioxidant[6,1], anticancer[1], anticomplementary[13] and antibacterial[10] effects. In clinical, *Trollius chinensis* Bunge. has been used for the treatment of tonsillitis, pharyngitis, enteritis etc[14]. *Flos Trollii* flowers are a rich source of flavonoids, including flavones[15] and flavones-C-glycosides[5,14,16], flavonol and flavono-O-glycosides, flavonone and flavonone-O-glycosides[17-18]. Among them, orientin and vitexin, as the main bioactive flavonoids in Flos Trollii, has some useful effects, such as antioxidant[5.19]and antiviral[1]etc.

In recent years, various new extraction techniques such as ultrasonic-assisted extraction, microwave-assisted extraction[20]and supercritical carbon dioxide extraction[21]have been established for the extraction of flavonoids from natural plants. Among them, ultrasonic-assisted extraction has shorter time, less solvent, higher extraction rate and better products and other advantage. Research of flavonoids in Flos Trollii is also ongoing. In the present study, one ultrasonic-assisted extraction was used for extract orientin and vitexin from Flos Trollii and the operational parameters were optimized using Response surface method (RSM). The Flos Trollii flavonoid extracted by different method were separated, identified and determined by HPLC-DAD-MS by using the vitexin and orientin as the standard reference. The objectives of this work were to investigate ultrasound-assisted extraction vitexin and orientin from Flos Trollii using a pilot-scale extraction and to study the influence of time, temperature and solvent-to-material ratio on vitexin and orientin yield for development and application of the resource. The research will be helpful to further exploit and utilize Flos Trollii as a medicinal resource.

EXPERIMENTAL SECTION

Trollius chinensis Bunge. (Flos Trollii) flowers were purchased in October 2013 from the Bayi drug market in Xinning, Qinghai Province of China, and were dried at 50℃ for 24h in an oven and then comminuted into pieces by a crusher and passed through a 40 mesh sieve and stored in darkness before analysis. Orientin and vitexin(both with purity≥99%) were purchased from Chengdu Must Bio-Technology Co.,Ltd. Chromatographic pure methanol was purchased from Shandong Yuwang Industrial Co.,Ltd. Before analysis, all solutions were filtered through a 0.22mm Nylon membrane (Alltech, Deerfield, IL, USA) and degassed.

Ultrasonic-assisted extraction (UAE)

The powdered Flos Trollii(2.0000g) was accurately weighed and soaked with 70% ethanol, and then placed in ultrasonic cleaner(Model KQ-200VDB, Kun Shan Ultrasonic Instruments Co., Ltd, Kunshan, Chian) and sonicated at 100W, 40KHz for different temperature, time and certain solid to liquid ratio. After extraction, the extract solution was removed the solvent with a rotary evaporator (Model R-210, BUCHI Laboratory Equipment Trading (Shanghai) Ltd. Shanghai, China) at 50℃.The concentrate was collected and stored at 4℃in darkness until further determination of vitexin and orientin and HPLC/MS analysis, the samples were prepared and analyzed in triplicate.

RSM design and data analysis

RSM combined with CCD was applied to optimize the extraction temperature (X1), time(X2) and solid to liquid ratio (X3) for ultrasonic-assisted extraction of vitexin and orientin from Flos Trollii. The coded variables equation showed in formula 1:

$$Y = \beta_0 + \sum_{j=1}^{k} \beta_j X_j + \sum_{j=1}^{k} \beta_{jj} X_j^2 + \sum \sum_{i<j} \beta_{ij} X_i X_j (k = 3) \quad (1)$$

Where k is the numbers of variables, β_0 is the constant term, β_i, β_{ii} and β_{ij} represents the coefficient of the first order terms, quadratic terms and interaction terms, respectively, while X_i and X_j are the independent coded variables.

During the entire experimental process, the extraction temperature varying from 35 to 55 ℃, time varying from 30min to 90min, and liquid to solid ratio varying from 10:1 to 20:1 were chosen based on former research(not list in here). All the experiments were repeated three times. A response surface analysis procedure (Design-Expert 7.1.3 Trial, State-Ease, Inc., Minneapolis MN, USA) was used for calculations and modeling of optimal conditions for ultrasonic-assisted extraction e of vitexin and orientin from Flos Trolliiin. Values of $P<0.05$ were regarded as significant.

Heating reflux extraction of flavonoids(HRE)

Ground dried Flos Trollii. (2.0000g) were extracted by 60mL 70% ethanol at 80℃ for 120min. The sample was extracted three times, and the extract was filtrated by Xinxing filter (Hangzhou Special Paper Industry CO., Ltd., Hangzhou, China). Then a rotary evaporator (Model R-210, BUCHI Laboratory Equipment Trading (Shanghai) Ltd. Shanghai, China) at 50℃ was used in order to remove solvent from the filtrate. The extract was collected and vacuum-dried at 50℃. After 15 min cooling, the dried extract was stored in 4℃ until further determination of vitexin and orientin of Flos Trollii flavonoids.

Determination of vitexin and orientin by HPLC-DAD and LC-MS/MS

Instruments and equipment

Experiments were performed using Agilent HP 1100 Series HPLC (Agilent, USA). The LC system consisted of an online vacuum degasser (model G1322A), a quaternary pump (model G1311A), an auto-sampler (model G1329A), a thermostated column compartment (model G1316A) and diode array detector (model G1315B). The mass spectrometer 1100 Series LC-MSD Trap-SL (ion trap) from Bruker Daltonik (Bremen, Germany) was equipped with an electrospray ionization (ESI) source.

HPLC-DAD analysis

The chromatographic separation of vitexin and orientin was achieved by using a reversed-phase waters symmetry C18 column (250×4.6 mm, 5μm, Waters Corp., Milford, USA) in conjunction with a gradient elution. The mobile phase consisted of water (solvent A) and pure methanol (solvent B), which was all filtered through a 0.22 mm Nylon membrane filter (Alltech, Deerfield, IL, USA). The initial composition of solvent B was 25% and was linearly increased to 35% at 30 min, to 40% at 40 min, to 50% at 50min. An aliquot of 20 μL solution was injected automatically. The chromatogram was monitored at 340 nm, and UV spectra of individual peaks were recorded in the range of 200–400 nm. Data were processed using Agilent Chemstation software. The flow rate was 1.0 mL/min

in a binary gradient mode, and temperature was 40□. Chromatographic peaks were identified by spiked the working standard with each individual flavonoid in turn.

HPLC-DAD-ESI-MS analysis
The mass spectrometer was equipped with an ESI source and controlled by Esquire-LC NT software. The ESI source was operated in positive-ion mode with a full scan mass range from m/z 150 to 800. The chromatographic separation condition was the same with the HPLC/DAD analysis mentioned above. The MS conditions were listed as follow: positive ion mode; gas (N2) temperature, 360℃; flow rate, 9.2 L/min; nebulizer pressure, 38 psi; capillary voltage, 3.8 kV; cone voltage, 40 V; corona current (nA), 4200 (pos).

Quantitative analysis of individual flavonoid, limit of detection (LOD), limit of quantity (LOQ) and recovery
Stock solutions of orientin (0.44mg/mL) and vitexin (0.46mg/mL) were prepared in methanol. Then, a set of mixed standard solutions were prepared by appropriate dilution of the stock solution with methanol, containing 6.88 to 220 μg/mL of orientin, 7.19 to 230 μg/mL of vitexin, All solutions were stored at 4℃ in darkness before analysis. The calibration curve of vitexin and orientin was prepared by plotting concentration(x, mg/mL) against its peak area (y) at λ=340nm. The regression equations and correlation coefficient (r) for each standard curve were automatically determined using Microsoft Excel software. The Flos Trollii flavonoids samples got by ultrasonic-assisted extraction with RSM (at each condition), heating reflux extraction were determined in triplicate.

The detection limits were estimated experimentally by injecting standard solutions of each flavonoids diluted in methanol until the signal-to-noise ratio for the standards reached a 3:1 ratio for LOD and a 10:1 for LOQ. Recovery experiments were performed to evaluate the accuracy of the methods. Three different concentrations of orientin and vitexin were added to known amounts, which where compared with Flos Trollii ultrasonic-assisted extraction without standards. The spiked samples were analyzed in triplicate by the established HPLC method. Accuracy was expressed as the percentage deviation between the amount of standard found by HPLC analysis and the amount added at the known concentrations.

Assessment of antioxidant activities
ABTS assay was based on the method of Re et al[22]with some modification. ABTS·+ reagent was produced by reacting 20 mL of 14 mM ABTS solution with 356 μL of 280 mM potassium persulfate (K_2SO_4) aqueous in the dark at room temperature for 15 h before use. The ABTS·+ solution was diluted to appropriate absorbance. 1.0 mL of different concentration sample (0.1-3.2 μg/mL), 2 mL of diluted ABTS ·+ solution and 1.0 mL of H_2O were consecutively added to react in the dark at room temperature for 30 min, and the absorbance at 734 nm was recorded. All the samples were tested three times.

Reducing power was determined according to the method of Wang et al[23]. Briefly, 1.5 mL of different concentration sample (0.1-3.2 μg/mL) was mixed with 0.5 mL of phosphate buffer (pH 6.6) and 0.5 mL of 1% (w/v) potassium ferricyanide solution. The mixture was incubated in a water bath at 50 °C for 20 min. Afterward, 1 mL of 10% (w/v) trichloroacetic acid solution was added, and the mixture was then centrifuged at 800 r/min for 10 min. A 3-mL aliquot of the upper layer was then combined with 0.5 mL of 0.1% (w/v) ferric chloride solution, and the absorbance was measured at 700 nm. All the samples were tested three times.

The flavonoids extract obtained by ultrasonic-assisted extraction on the optimization condition was subjected to analysis of their anti-oxidant activity by using a 2,2-diphenyl-1-picrylhydrazyl(DPPH)radical-scavenging assay. This method previously described by Amarowicz et al[24]was used with slight modifications in order to assess the DPPH• free radical scavenging capacity of flavonoids from Flos Trolliioils. The absorbance was measured at 517 nm and all the experiments were repeated three times. Briefly, 1mL of different concentration sample (0.1-1.8 μg/mL) and added to 5 mL of 0.025% DPPH (Sigma-Aldrich) in methanol. The mixture was shaken vigorously and incubated in dark for 30 min, and then placed in an UV759 spectrophotometer (Shanghai Precision & Scientific Instrument Co,.Ltd, Shanghai, China) to monitor the absorbance at 517 nm. The radical-scavenging activities of samples, expressed as percentage of DPPH• scavenging effect.

RESULTS AND DISCUSSION

HPLC separation
A HPLC method for the analysis of flavonoids was established. In order to select an appropriate detection method, the DAD detection wavelength(254nm, 270nm, 320nm, 340nm) along with the gradient program, flow rate and column temperature were be optimized. Under optimum conditions, the method provided repeatable and good separation within 50 min for samples (UAE, HRE). As shown in Fig.1, all peaks were got good separation.

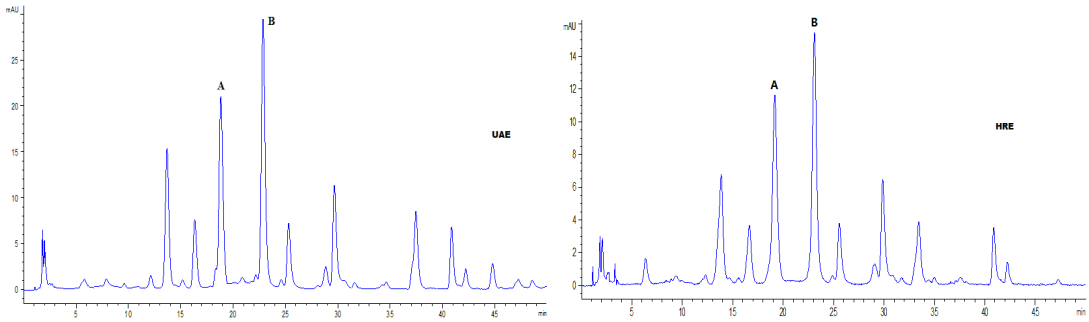

Fig.1. Comparison of HPLC chromatograms at 340 nm of two different samples (UAE and HRE, A stand for orientin, B stand for vitexin)

Flavonoid identification by HPLC-DAD-ESI-MS

By comparing the retention times and UV-Vis spectra (Fig.2) of the two standards in the same chromatographic conditions, we found that the samples obtained by the different extraction methods all contained the two flavone C-glycosides (orientin, vitexin). The ionization and fragmentation of the two components agree well with this conclusion. As expected, every flavonoid component produced an intense molecular ion peak at *m/z* $(M+H)^+$ (in ESI-MS spectrum). The selected reaction monitoring was based on the m/z $(M+H)^+(449.4)\rightarrow431.0$ transition for orientin; $(M+H)^+(433.4)\rightarrow415.0$ transition for vitexin. In the ESI-MS/MS spectra of the two flavone C-glycosides, the fragment ions $(M+H-150)^+$, $(M+H-120)^+$ and $(M+H-96)^+$ were observed, implying that the glucoside had split up.

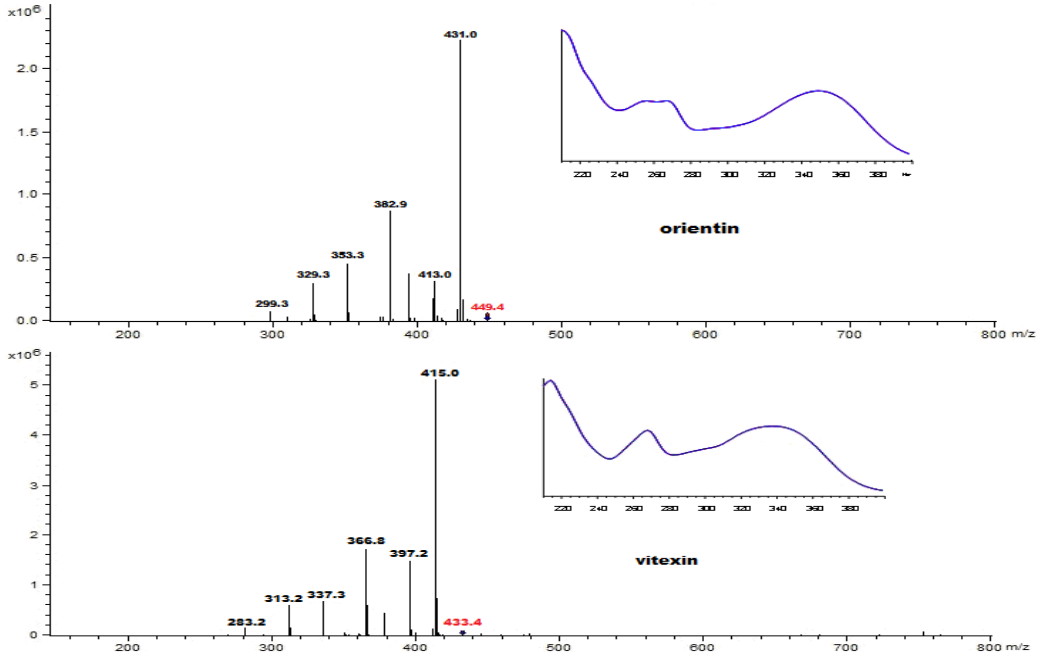

Fig.2. The UV and positive mode ESI–MS/MS spectrum for the two detected flavonoids

Quantitative analysis of flavonoids from HRE and UAE

Two flavonoids in *Trollius chinensis* Bunge. extracted by two different methods (UAE, HRE) were quantitatively determined using the method developed here. As showed in Table-1, all calibration curves exhibited good linear regression, being higher than 0.9990, and the ranges of the curves were adequate for analyzing the two flavonoids.

To establish the reliability of the HPLC method, the recoveries of the two standards were determined by adding known amounts of standards to the UAE sample under the same conditions as shown in Table-2. All recoveries were in the range of 96.43-98.90%, suggesting the method is reliable.

Table-1.Linear Regression Equation, Correlation Coefficient and LOD for Flavonoids Quantification by HPLC-UV/DAD

Standard	Retention time(min)	Regression equation	correlation coefficients	Linearity range (μg/mL)	LOD (ng/mL)	LOQ (ng/mL)
orientin	19.012	y=7.8894x+0.1021	0.9995	6.88-220	45.87	137.61
vitexin	22.913	y=15.2340x+0.0913	0.9993	4.80-154	43.64	130.91

Table-2.Recovery (%) Test of Two Flavonoids for this Method (n=3)

Standard	Original mean(mg)	Quantity added(%)	Spiked mean(mg)	Detected mean(mg)	Recovery(%)	RSD(%)
orientin	0.35	80	0.28	0.62	96.43	2.67
		100	0.35	0.69	97.14	1.38
		120	0.42	0.76	97.62	1.72
vitexin	1.52	80	1.22	2.72	98.36	1.87
		100	1.52	3.01	98.03	2.31
		120	1.82	3.32	98.90	1.46

Recovery(%)=($amount_{determined}$ - $amount_{original}$)/$amount_{spiked}$×100%

Optimization of extraction procedure

The RSM with CCD was used to evaluate the extraction parameters and optimize experimental conditions in the ultrasonic-assisted extraction process. The vitexin and orientin yield under different extraction conditions are presented in Table-3.

As shown in Table-3, the yield of vitexin was from 1.08% to 1.25%, orientin was from 0.53% to 0.62% for UAE. The yield of HRE was 0.89% 0.52%. Thus, the yield of vitexin and orientin for UAE was more efficient than HRE.

Table-3. Experimental Scheme and Results Obtained from RSM for the Vitexin and Orientin Yield

No.	time (X1, min)	temperature (X2,℃)	liquid to solid ratio (X3, g/mL)	vitexin yield (%)	orientin yield (%)	Sum vitexin and orientin(%)
1	1(90)	1(55)	1(20)	1.23	0.61	1.84
2	1	1	-1(10)	1.20	0.58	1.78
3	1	-1(35)	1	1.19	0.59	1.78
4	1	-1	-1	1.13	0.56	1.69
5	-1(30)	1	1	1.21	0.60	1.81
6	-1	1	-1	1.14	0.57	1.71
7	-1	-1	1	1.11	0.55	1.66
8	-1	-1	-1	1.07	0.53	1.60
9	-1.682(9.5)	0	0(15)	1.17	0.59	1.76
10	1.682(110.5)	0	0	1.25	0.62	1.87
11	0(60)	1.682(61.8)	0	1.09	0.55	1.64
12	0	-1.682(28.2)	0	1.18	0.60	1.78
13	0	0(45)	1.682(23.4)	1.08	0.54	1.62
14	0	0	-1.682(6.6)	1.19	0.60	1.79
15	0	0	0	1.22	0.61	1.83
16	0	0	0	1.22	0.61	1.83
17	0	0	0	1.24	0.62	1.86
18	0	0	0	1.23	0.62	1.85
19	0	0	0	1.23	0.61	1.84
20	0	0	0	1.23	0.61	1.86
HRE	120	80	40	0.89	0.52	1.41

Regression modeling of UAE

The vitexin and orientin yield obtained in all the CCD experiments are listed in Table -3. Experimental results were analyzed using RSM. The second-order polynomial equation that fitted the coded variables was from previous studies. The regression model for the relationship between vitexin and orientin yield (Y) and the uncoded values of independent variables of X_1, X_2 and X_3 and their interactions is shown in the following equation:

$$Y=-0.1637+4.9060\times10^{-3}X_1+0.0507X_2+0.0684X_3-4.5833\times10^{-5}X_1X_2-8.3333\times10^{-6}X_1X_3-2.5000\times10^{-5}X_2$$

$$X_3-1.2586\times10^{-5}X_1^2-4.8451\times10^{-4}X_2^2-2.0087\times10^{-3}X_3^2$$

The analysis of variance (ANOVA) for the response surface model is given in Table-4. All quadratic parameters were significant at the level of $P < 0.01$. Model predictions of vitexin and orientin yield were adequate, as indicated by the error analysis that showed a non-significant lack of fit ($P>0.05$). The regression model for vitexin and orientin yield was highly significant ($P<0.01$). The value of R^2 (0.9825) indicated that the experimental data were in good agreement with predicted values of yield. The F-value for the lack of fit was also insignificant ($P > 0.05$), meaning

that this model was sufficiently accurate in predicting the relevant responses.

Table-4.ANOVA Analysis of Predicated Second-Order Polynomial Model for the Response Variable

Variables	Degree of freedom	Sum of Squares	Mean squares	*F*-value	*P*-value
Quadratic Model	9	0.140	0.016	62.46	<0.0001
X1	1	0.018	0.018	5.88	<0.0001
X2	1	0.031	0.031	23.73	<0.0001
X3	1	0.026	0.026	21.70	<0.0001
X1×X2	1	1.513×10^{-3}	1.513×10^{-3}	4.50	0.0334
X1×X3	1	1.250×10^{-5}	1.250×10^{-5}	18.01	0.8272
X2×X3	1	1.250×10^{-5}	1.250×10^{-5}	8.00	0.8272
X1×X1	1	1.849×10^{-5}	1.849×10^{-5}	0.34	0.0213
X2×X2	1	0.034	0.034	135.93	< 0.0001
X3×X3	1	0.036	0.036	146.03	< 0.0001
Lack of fit	1.539×10^{-3}	5	3.078×10^{-4}	1.62	0.3048
Pure error	9.500×10^{-4}	5	1.900×10^{-4}		
Total error	0.14	19			

Response surface analysis

Multiple regression coefficients, obtained by employing a least squares technique to predict a second-order polynomial model for vitexin and orientin yield, are summarized in Table-4. As shown in Table-4, all three factors had a notable effect on yield. There was significant interaction ($P < 0.05$) between X_1X_2, but no significant interaction ($P > 0.05$) with X_1X_3 and X_2X_3 within the experimental range. The results indicate that linear, quadratic and interactive effects of the independent variables may be the primary determining factors of vitexin and orientin yield. Three-dimensional shaded surfaces of the second-order polynomial model were used to predict the interactive effects of operational parameters for the vitexin and orientin of UAE (Fig 3-5).

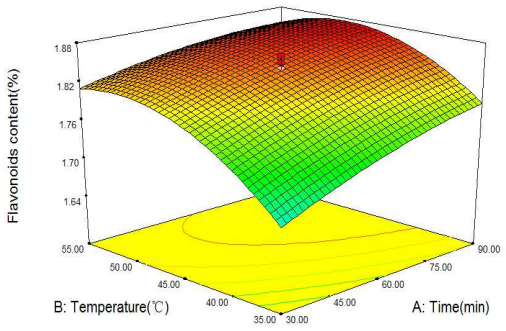

Fig.3. Response surface for the effect of time and temperature on the vitexin and orientin yield

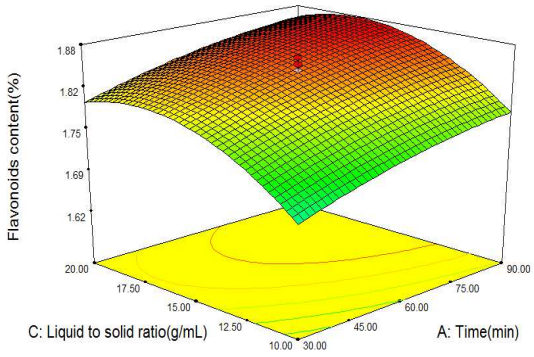

Fig.4. Response surface for the effect of time and liquid to solid ratio on the vitexin and orientin yield

Fig.3 shows the three-dimensional response surface (a) and contour plots (b) showing the effect of time and temperature on yield. The extraction temperature is one of the main parameters affecting yield. At a given temperature, yield increases with extraction time, especially for short durations and low temperatures. Temperature had a positive linear effect on yield for short intervals of extraction. This is most likely due to the improvement of flavonoid solubility from the increased mass transfer rate. However, if the temperature is higher than a given threshold (about 49°C), flavonoids start to break down. Fig.4 shows the effect of extraction time and solid-to-liquid ratio on the vitexin and orientin. Figure 4 illustrates the importance of extraction time for UAE. An increase in yield

with increasing time in the early stages of extraction is evident. As shown in Fig.5, yield significantly increases with increasing solid-to-liquid ratio. This ratio is another important parameter affecting UAE yield. From 10 g/ml to 20 g/ml, yield increases sharply, suggesting that vitexin and orientin are more efficiently extracted with more solvent. Although increasing this ratio boosts yield, a higher ratio entails greater costs and is not conducive to commercial production.

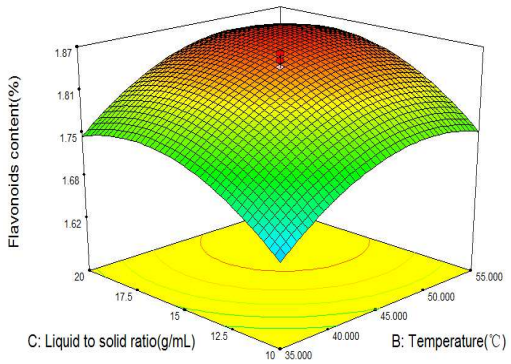

Fig.5. Response surface for the effect of temperature and liquid to solid ratio on the vitexin and orientin yield

Optimization and verification of optimized models

The optimal conditions predicted by the model were as follows: time of 71.18 min, temperature of 48.54°C and solid-to-liquid ratio of 16.57 g/ml, where the predicted yield reached a maximum of 1.87%. For convenience, these parameters were altered slightly with little effect on yield. Thus, an extraction time of 70 min, temperature of 48°C and solid-to-liquid ratio of 16 g/ml were deemed optimal. Verification of these results showed that the predicted values from the model were reasonably close to observed values.

Assessment of antioxidant activities

After oxidation, ABTS was transferred to $ABTS^{\cdot+}$. When the corresponding antioxidant compound was added, color of $ABTS^{\cdot+}$ reagent would fade. ABTS scavenging activities for the flavonoids extracted from Flos Trollii were similar. With different concentrations (0.1-13.2 μg/mL), this activity increased with increasing amount of flavonoids(Fig.6).

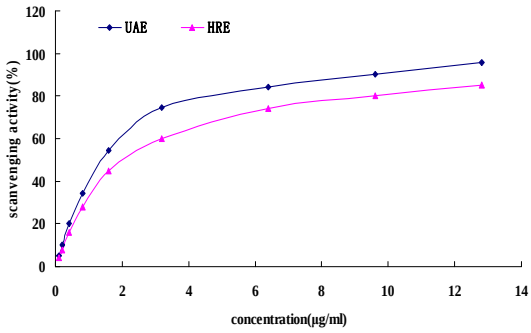

Fig.6. Antioxidant activities of flavonoids extracted from *Trollius chinensis* Bunge.: ABTS scavenging activities

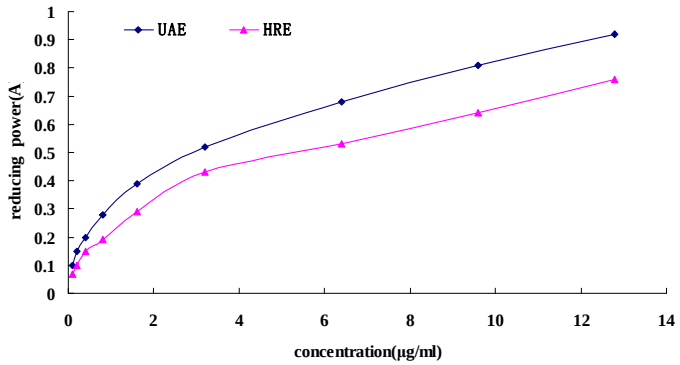

Fig.7. Antioxidant activities of flavonoids extracted from *Trollius chinensis* Bunge.: the reducing power

The reducing power, which may serve as a signification reflection of the antioxidant activity, was determined by reduction of the Fe^{3+} to Fe^{2+}. Increased absorbance of the reaction mixture indicates greater reducing power. Fig. 7 showed the comparison for the reducing power of flavonoids extracted from Flos Trollii. All samples showed some degree of reducing power. The reducing power increased with increasing amount of falvonoids.

The antioxidant activity of flavonoids extracted by UAE and HRE were also tested by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging assay. As showed in Fig. 8, the decrease in the concentration of flavonoids resulted in the reduction of its antioxidant ability, the antioxidant activity of the flavonoids was found to be concentration-dependent. However, under the same condition, the antioxidant activity of flavonoids obtained by HRE was lower than UAE.

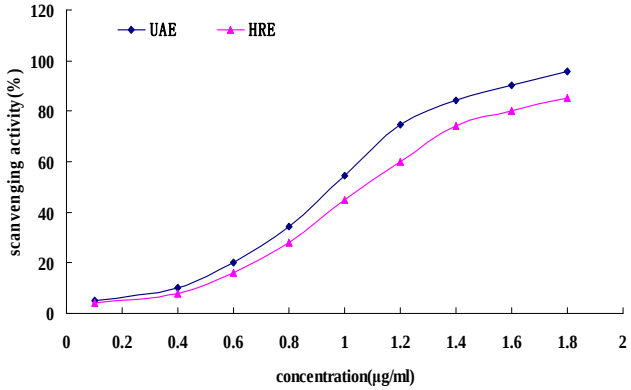

Fig. 8. Antioxidant activities of flavonoids extracted from *Trollius chinensis* Bunge.: DPPH scavenging activities

CONCLUSION

In the study, flavonoids from *Trollius chinensis* Bunge. were extracted by different method(UAE, HRE). Meanwhile, simultaneous determination of two flavonoids (orientin and vitexin) was accomplished by a useful, rapid and simple HPLC-DAD-ESI-MS/MS method. CCD and RSM were applied for modeling and the prediction of the orientin and vitexin extraction yield. The optimum extraction conditions found by maximizing the dependent variables were time of 70min, temperature of 48□and liquid to solid ratio of 16g/mL, where 1.87% yield was predicted. This is the first report of a RSM and HPLC/MS method to simultaneous determined the two main flavonoids in *Trollius chinensis* Bunge.. The assay is reproducible, sensitive and has been fully validated. The results are valuable for further utilizing and development of *Trollius chinensis* Bunge.

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