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Research Article

Optimization of ultrasound-assisted extraction of flavonoids from *Emilia prenanthoidea* DC. using response surface methodology and exploration of the ecological factors on total flavonoid and antioxidant activity

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Abstract: *Emilia prenanthoidea* DC. (EP) is a medicinal plant that belongs to the tribe Emilia Cass in the family Asteraceae. Although it has a long history of medicinal use, there are few research reports on this herb. In this study, for the first time, ultrasound-assisted extraction (UAE) conditions were optimized by response surface methodology (RSM) to extract total flavonoids from EP. An optimized method was used to determine the total flavonoid content (TFC) and antioxidant activity of EP extracts from 12 regions in Guizhou, China. Combining environmental factors in 12 regions, the effects of different growth environments on TFC and antioxidant activity of EP extracts were analyzed. In this work, it is proven that the optimal conditions for extraction are a solvent concentration of 45%, an ultrasonication time of 25 min, and a liquid-solid ratio of 46.25 mL/g. Under this condition, the highest TFC (41.182 mg/g) was found in the EP extract from Daping, while the lowest TFC (28.865 mg/g) was found in the EP extract from Liping. Evaluation of antioxidant activity showed that the highest antioxidant capacity was found in EP extracts from Daping, followed by Tuan Shan, Yaobai and Green Lake. Ecological factors affected both total flavonoids and antioxidant activity in EP. Annual sunshine hours in different regions had a significant effect on TFC in EP (r = −0.841, P < 0.01). In conclusion, this study established an effective method for the extraction of total flavonoids from EP, an effective natural antioxidant. The effects of the growth environment on the TFC and antioxidant capacity were also analyzed. It provides an experimental basis for the extensive utilization of EP.

Keywords: *Emilia prenanthoidea* DC.; response surface methodology; flavonoids; antioxidant activity; ecological factors

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1 Introduction

A series of physiological processes in humans will produce oxygen-centered free radicals and other reactive oxygen species as by-products. However, excessive free radicals and other reactive oxygen species in the body will cause a variety of diseases, such as atherosclerosis[1], Alzheimer's disease[2], cancer[3] and so on. Antioxidants protect the body from free radicals and other reactive oxygen species, thereby regulating oxidative stress and delaying the onset of some chronic diseases[4]. Antioxidants are also widely used in the food industry for their ability to prevent the oxidation of substances. The synthesized antioxidant compounds have a variety of side effects[5], for example, butylated hydroxytoluene has been reported to be carcinogenic. In recent years, people have paid more and more attention to natural sources of antioxidants to replace synthetic antioxidant compounds, such as fruits and vegetables and Chinese medicinal herbs containing a large number of antioxidant components[6].

Flavonoids are widely found in the plant kingdom[7]. The phenolic hydroxyl group in flavonoids has a certain ability to capture oxygen free radicals which can be used as a natural antioxidant to prevent a variety of diseases in the human body. Total flavonoids are important secondary metabolites of photosynthesis and have various pharmacological activities, such as antioxidant, anti-inflammatory, anti-tumor, anti-virus, antibacterial activity, etc. They exist widely as bioactive constituents in TCM[8-10]. Studies have shown that TCM are effective in treating anti-tumors. Regulation of apoptosis is one of the main mechanisms of Chinese medicine against tumors, and flavonoids can induce apoptosis.

Emilia prenanthoidea DC. (EP) occurs in Yunnan, Guizhou,

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Guangdong, Guangxi, Zhejiang, Fujian, and other provinces in China. It is also distributed from India to the central and southern peninsulas. As a TCM, EP having a long history and widely used in a broad spectrum of indications. EP is well-known for its effects in treating heat stress, diarrhea, bruises, and poison[11]. *Emilia sonchifolia* is one of the main components of Chinese patent medicine Huahong Tablets and has been recorded in the Chinese Pharmacopoeia. *E. sonchifolia* and EP are virtually indistinguishable in morphology, similar in composition, and have proved to be identical in some of their pharmacological effects[12-14]. It can be seen that the pharmacological effect of EP should not be underestimated, but the practical application of EP is few at present, and the related research reports are also few.

It has been shown that flavonoids are the characteristic chemical constituents of EP[11]. So far, few studies on the extraction process of total flavonoids and antioxidant effects in EP have been reported. Ultrasound-assisted extraction (UAE) has been used to extract various plant flavonoids due to its high efficiency, ease of operation, time-saving, and solvent-saving features[15-17]. The extraction process is influenced by many factors and the right parameters are key to improving efficiency. In this study, the extraction of total flavonoids in EP was optimized using a Box–Behnken design (BBD) of RSM. The experimental conditions tested included solvent concentration, material-liquid ratio, and ultrasonication time. The optimized methodology was used to examine contents of total flavonoids and antioxidant capacity of EP from 12 different regions. This study represents the first systematically optimized comparison of EP flavonoid content, antioxidant capacity, and their modulation by ecological factors.

2 Materials and methods

2.1 Reagents and materials

Ascorbic acid was obtained from Guizhou DiDa Technology Co., Ltd. (Guiyang, China). 1,1-Diphenyl-2-picrylhdrazyl (DPPH) was acquired from Kasei Kogyo Co., Ltd. (Tokyo, Japan). 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) was obtained from Lark Technology Co., Ltd. (Beijing, China).

2.2 Sample collection and UAE process

EP samples were collected from 12 origins in the Guizhou province of China , and samples were authenticated by Professor Qingwen Sun (Guizhou College of Traditional Chinese Medicine, Guiyang, China). Data collected on ecological factors for each region is available from http://hgk.guizhou.gov.cn/index.vhtml. All EP samples used were whole plants.

Dried EP from each origin was processed into powder by 60 mesh, using a Chinese medicine crusher from Wenling Da Machinery Co., Ltd. (Taizhou, China). The powdered sample of EP (0.2 g) was mixed with a specified volume and concentration of ethanol. The mixture was kept for a certain time in an ultrasonic extraction reaction workstation manufactured by Kunshan Ultrasonic Instrument Co., Ltd. (Kunshan, China). The extraction power is 300 W and the frequency is 40 MHz. After cooling to room temperature, solvent was added to offset the lost weight. After centrifugation at 4,000 r/min for 10 min, the supernatant was stored in sealed dark bottles. The filtrate was stored at 4 °C before the experiment.

2.3 Determination of total flavonoids

The total flavonoid content (TFC) of the EP samples was determined by the method of Varinskii *et al.*[18] with slight modification. Briefly, 1 mL of extract solution was mixed with 1.5 mL 5% $NaNO_2$ into a 10 mL Erlenmeyer flask standing for 5 min. Then, 0.5 mL 10% $Al(NO_3)_3$ was added. After another 5 min standing, 4 mL 4% NaOH was added. The volume of the total mixture was made up to 10 mL with distilled water. After 5 min of incubation, the absorbance was measured at 510 nm. All tests were performed in triplicate. Using rutin as a control, the standard curve was determined to be $y = 6.145,8x - 0.008,8$ ($r^2 = 0.999,1$), and the measured results were calculated according to the following equation (Eq. 1).

$$\text{The TFC yield } (\text{mg/g}) = \frac{C \times V \times N}{M} \quad (1)$$

where C is the TFC calculated from the standard curve (mg/g), N is the fold-dilution of the sample, V is the total volume of the extract (mL), and M is the total weight of the EP sample (g).

2.4 Response surface experiment design

The design of response surface methodology (RSM) was carried out to optimize the method for the determination of total flavonoids with three factors. The preliminary range of extraction variables was obtained by single-factor experiments, and the best combination for extraction was determined by a three-level, three-factor BBD of RSM. Based on the results of the single-factor experiment, three independent variables were determined as shown in Table 1, including solvent concentration (X_1, %), ultrasonic extraction time (X_2, min), and mass-volume ratio (X_3, g/mL). The independent variables were assigned to three levels, coded as +1 (high level), 0 (medium level), and −1 (low level). Using a full quadratic equation (Eq. 2) to investigate interactive impacts on variables by the data obtained from the process above.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i>j}^{k} \beta_{ij} X_i X_j \quad (2)$$

where Y is the response variable to predict TFC; β_0 is the constant, β_i, β_{ii}, and β_{ij} are regression coefficients of linear parameter, second order, and interaction coefficients, respectively. X_i and X_j are encoded independent variables ($i > j$). k equals the number of the tested factors ($k = 3$).

Table 1 Coded values and the corresponding actual values of the optimization parameters

Solvent	Code	Solvent concentration (%)	Ultrasonic extraction time (min)	Liquid-solid ratio (mL/g)
Methanol	−1	20	10	30
	0	40	20	40
	1	60	30	50

2.5 Antioxidant activity

2.5.1 *Measurement of antioxidant activity with DPPH*

Antioxidant activity against DPPH free radicals was determined as described by Chen *et al.*[19] with slight modifications. Briefly, 0.5 mL diluted sample was added to 0.5 mL DPPH (250 mol/L) solution and left at room temperature for 40 min. The absorbance of the solution was measured at 517 nm in the dark. The effect of the sample on DPPH free radical scavenging was calculated as follows (Eq. 3):

$$\text{DPPH scavenging effect}\ (\%) = \frac{(A_0 - A_1)}{A_0} \times 100 \qquad (3)$$

where A_0 is the absorbance of DPPH working solution after mixing with ethanol, and A_1 is the absorbance of DPPH working solution after mixing with the sample.

2.5.2 *Measurement of antioxidant activity with ABTS*

The ABTS Robin's method was used to determine the antioxidant activity of EP extracts with some modifications[20]. $ABTS^+$ was prepared by reacting 7 mmol/L ABTS solution with 2.45 mmol/L potassium persulfate in the dark at room temperature for 12 h. The measurement was performed by formulating a series of EP sample solutions with ethanol. Anhydrous ethanol was used as the blank control. Here, 200 μL of each concentration of the sample solution was mixed with 0.8 mL $ABTS^+$ working solution. After reacting at room temperature for 20 min, the absorbance was measured at 734 nm. Then, the clearance rate was calculated using the following formula (Eq. 4):

$$\text{ABTS inhibition effect}\ (\%) = \frac{(A_0 - A_1)}{A_0} \times 100 \qquad (4)$$

where A_0 is the absorbance of ABTS working solution after mixing with absolute ethanol, and A_1 is the absorbance of ABTS working solution after mixing with the sample.

2.5.3 *Measurement of antioxidant activity with ferric reducing/antioxidant power (FRAP)*

The FRAP method for determining the total antioxidant capacity is a spectrophotometric method for evaluating the effectiveness of an antioxidant for Fe^{3+}-tripyridyltriazine (TPTZ) complex reduction to its Fe^{2+}-tripyridyltriazine form. The latter has an intense blue color that absorbs light at 593 nm. For the reduction of Fe^{3+}, we used the method described by Benzie *et al.*[21] with some modifications. Briefly, a fresh FRAP working solution was prepared by mixing 10 mmol/L TPTZ solution (dissolved with 40 mmol/L hydrochloric acid), 20 mmol/L of $FeCl_3$ solution, and 0.3 mol/L of acetic acid buffer (pH 3.6) at a volume ratio of 1:1:10. The sample was dissolved at an appropriate concentration with absolute ethanol. Then, 200 μL of the sample was added to 0.8 mL of freshly prepared FRAP working solution, mixed, and reacted at 37 °C for 40 min. Then we measured the absorbance at 593 nm. A standard curve was drawn with a 25–1,000 μmol/L Trolox standard solution. FRAP of the sample was expressed as the Trolox equivalent antioxidant capacity (TERC) value which was equivalent to the micromolar number of Trolox (μmol/L TERC).

2.6 Statistical analysis

The experiments were all repeated three times. Regression coefficients were determined by analysis of variance (ANOVA). Statistical analysis was performed using the software Design-Expert 7.1. Pearson's correlation analysis (SPSS 17.0 software, SPSS Inc., USA) was used for evaluating the correlation between the TFC and the antioxidant capacity.

3 Results and discussion

3.1 Optimization of extraction conditions

3.1.1 *Single-factor investigation of extraction method*

The extraction effect is affected by a number of factors. The extraction effects of three solvents, water, ethanol and methanol, have been compared by one-way experiments prior to the experiment. It was found that ethanol extraction was more effective. On this basis, the one-way experiment was continued to determine the optimum extraction process of total flavonoids from EP extracts. The effects of solvent concentration (0–100%), liquid-solid ratio (1:10–1:50) and ultrasonic extraction time (10–50 min) were investigated using the controlled variable method. The conditions used were a solvent concentration of 40%, a liquid-solid ratio of 1:40, and an extraction time of 20 min.

As can be seen from Fig. 1, solvent concentration, liquid-solid ratio and sonication time had a significant effect on the TFC. For solvent concentration, there was no significant increase in TFC with increasing solvent concentration when the solvent concentration exceeded 40%. The effect of liquid-solid ratio on TFC showed that the liquid-solid ratio (from 1:10 to 1:40) was directly proportional to the TFC. However, the TFC began to decrease after the liquid-solid ratio exceeded 1:40. For ultrasound time, there was no significant increase in TFC with time after 20 min. Therefore, the results of 40%, 20 min, and 40 g/mL were the best single-factor extraction conditions.

3.1.2 *Extraction model and statistical analysis*

The BBD of the RSM in the experimental design involved three independent variables, three levels, and five center point repeats which were all used to evaluate the stability of the extraction process. The 17 experimental conditions and results in the BBD design scheme are shown in Table 2. All tests were performed in triplicate. The actual yields ranged from 28.865 mg/g to 41.128 mg/g.

The three experimental variables (X_1: solvent concentration, X_2: ultrasonic time, and X_3: liquid-solid ratio) had a significant effect on the EP extraction rate (Table 3). The prediction model of the extracted yield value can be represented by a second-order polynomial equation. The quadratic equation obtained is as follows (Eq. 5):

$$Y = 23.24 + 0.63X_1 + 0.63X_2 + 0.96X_3 - 0.14X_1X_2 - 0.12X_1X_3 + 0.61X_2X_3 - 1.44X_1^2 - 0.71X_1^2 - 1.09X_3^2 \qquad (5)$$

The extraction rate prediction values based on the above-described secondary prediction model are shown in Table 2. The results of the statistical regression curve by T test, F test, and ANOVA are shown in Table 3. The F value of the model is 212.73, $P < 0.000,1$, indicating that the model had high statistical significance. Furthermore, the deterministic coefficient R^2 was 0.996,4 which is close to 1 suggesting that the measured values are very close to the predicted values of the model. The value of the adjusted determination coefficient R^2 (adj R^2) was 0.991,7, which indicates that the total flavonoid extracting response in EP was 99.2% and can therefore be predicted by this model. Only 0.8% of the variability could not be explained by this model, indicating that the model has a high degree of fit.

The P value is used as a scale to measure the interactive influence of each coefficient and also to indicate the interaction between variables. Here, the smaller the P value, the more meaningful the correlation coefficient. As seen in Table 3, the linear equations' one-time coefficients (X_1, X_2, X_3), quadratic term coefficients (X_1^2, X_2^2, X_3^2), and quadratic cross product terms (X_2X_3) had significant differences $P < 0.05$. This suggested that these three extraction conditions-solvent concentration, extraction time, and solid-liquid ratio had a significant effect on the

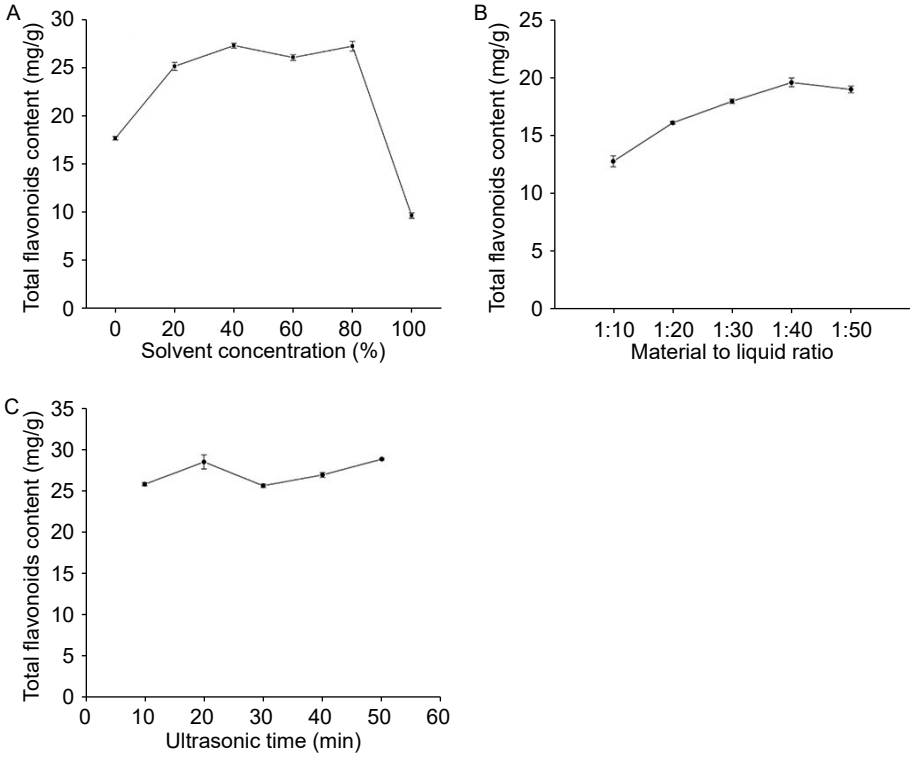

Figure 1 Effect of different solvent concentration (A), liquid-solid ratio (B) and ultrasonic time (C) on TFC in EP.

Table 2 Coded experimental and predicted values for the RSM design using methanol as the solvent

Run	X_1	X_2	X_3	Content (mg/g)	
				Experimental	Predicted
1	0	0	0	23.636	23.44
2	−1	−1	0	19.891	19.89
3	1	−1	0	21.478	21.48
4	0	−1	1	21.328	21.33
5	0	−1	−1	20.611	20.61
6	−1	0	−1	19.237	19.24
7	0	0	0	23.448	23.44
8	0	1	−1	20.713	20.71
9	0	0	0	23.458	23.44
10	0	0	0	23.459	23.44
11	1	0	1	22.333	22.37
12	0	0	0	23.183	23.18
13	1	0	−1	20.689	20.69
14	0	1	1	23.876	23.88
15	1	1	0	22.406	22.41
16	−1	1	0	21.361	21.36
17	−1	0	1	21.367	21.37

Note: X_1, solvent concentration; X_2, ultrasonic time; X_3, liquid-solid radio.

extraction of total flavonoids. At the same time, the extraction time and the ratio of material to liquid also had an interaction with a significant effect on the extraction of total flavonoids.

The 3D response surface and 2D contour plots are graphical forms of the corresponding surface quadratic regression equation. This format makes it easier to visually appreciate the relationship between the response values and the experimental values. The contour plots reflect the interaction of the various factors on the response value. A circle indicates that the two-factor interaction was not significant, while an ellipse indicates that the two-factor interaction was significant. In this study, the extraction rate of total flavonoids of EP was determined by the ratio of material to liquid, solvent concentration, and ultrasonic time, as shown in Fig. 2. The extraction time and the ratio of material to liquid changed with these two factors. Figure 3D shows a steep change and Fig. 2D shows a clear oval shape, suggesting that these two factors have the most significant interaction effect on the extraction of total EP flavonoids.

To verify the accuracy of the experimental model equations, a verification test was carried out under the following experimental conditions: solvent concentration: 43.23%, ultrasonic extraction time: 26.94 min, liquid-solid ratio: 46.24 mL/g. For an optimal result, experimental values should be in accordance with predicted values from the model equations. In order to assess whether a predicted value deviates from the actual experimental value, the experiment was conducted using the optimal conditions of the model equation. The TFC in the EP extract obtained from the actual experimental extraction was (23.82 ± 0.03) mg/g (n = 3). The relative error between the experimental value and the theoretical value was 1.47%. This analysis indicated that the experimental values coincided with predicted ones, and the equations accurately predicted test results. The verification test results are shown in Table 4. Combined with the ease of the ultrasonic extraction method, the optimized extraction process is more efficient than alternative processes. We therefore propose this approach as an improved method for the industrial extraction of total flavonoids from EP.

3.2 The TFC of EP from 12 regions

Extraction was performed using the optimized extraction method to determine the content of total flavonoids from 12 regions. Comparing the TFC, it was found that there was a significant difference in TFC across the 12 regions (P < 0.05). The

Table 3 ANOVA on Box-Behnken design

Source	Sum of squares	Degree freedom	Mean square	*F*-value	*P*-value	Significant
Model	32.88	9	3.65	212.73	< 0.000,1	Significant
X_1	3.19	1	3.19	185.60	< 0.000,1	
X_2	3.19	1	3.19	185.45	< 0.000,1	
X_3	7.32	1	7.32	426.36	< 0.000,1	
X_1X_2	0.07	1	0.07	4.28	0.077,5	
X_1X_3	0.06	1	0.06	3.44	0.106,1	
X_2X_3	1.50	1	1.50	87.08	< 0.000,1	
X_1X_1	8.72	1	8.72	507.73	< 0.000,1	
X_2X_2	2.14	1	2.14	124.85	< 0.000,1	
X_3X_3	5.01	1	5.01	291.87	< 0.000,1	
Residual	0.12	7	0.017			
Lack of fit	0.02	3	0.003	0.19	0.897,4	Not significant
Pure error	0.11	4	0.026			
Corrected total	33.00	16				
R^2	0.996,4	9	3.65	212.73	< 0.000,1	Significant
Adj R^2	0.991,7					
Predicted R^2	0.987,7					
Adequate precision	46.105					

Note: X_1, solvent concentration; X_2, ultrasonic time; X_3, liquid-solid radio.

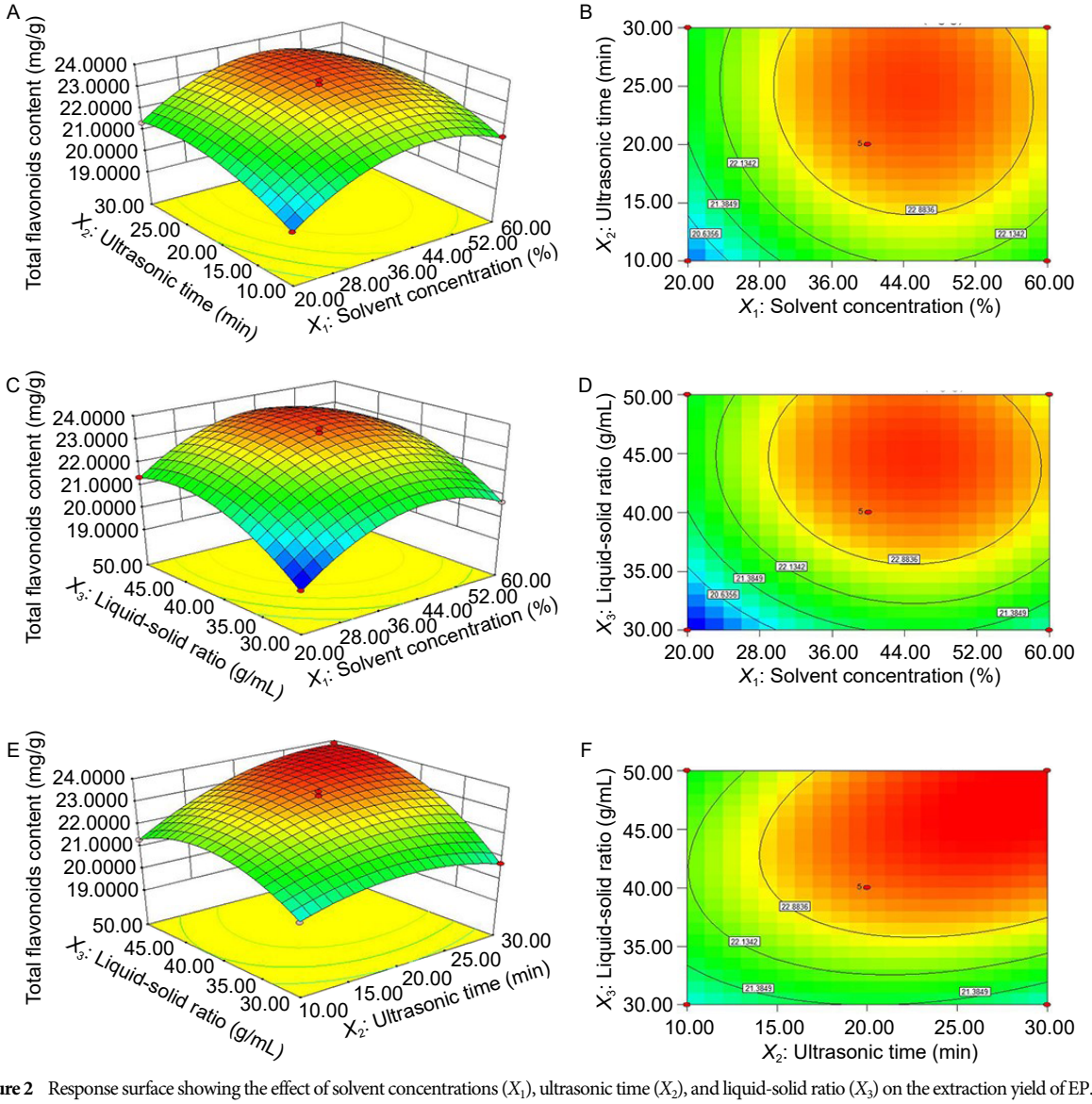

Figure 2 Response surface showing the effect of solvent concentrations (X_1), ultrasonic time (X_2), and liquid-solid ratio (X_3) on the extraction yield of EP.

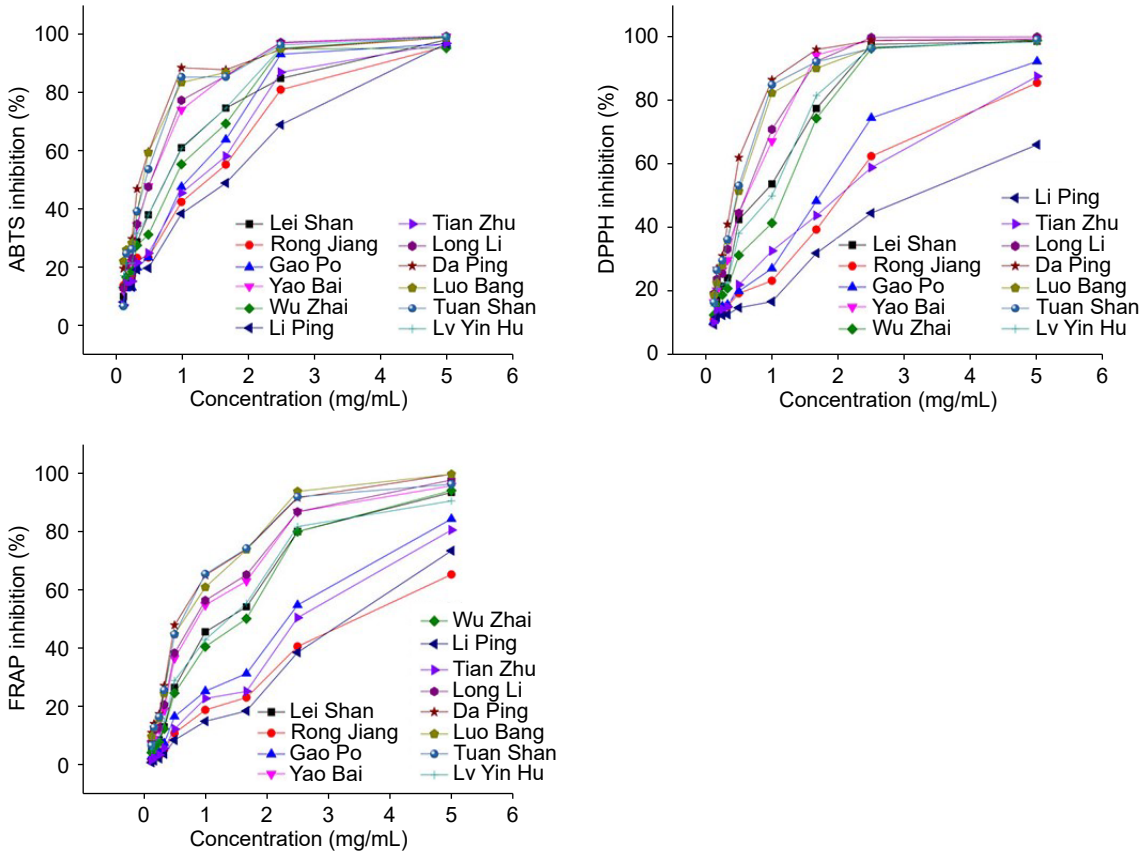

Figure 3 ABTS clearance rate, DPPH clearance rate and the total reduction capacity of extracts from 12 origins.

Table 4 Results of the model validation experiments

No.	Optimum conditions				Extraction yield (mg/g)	
	The quantity of sample (g)	Solvent concentration (%)	Ultrasonic time (min)	Liquid-solid ratio (mL)	Experimental	Predicted
1	0.203,9	45.00	25.00	9.25	23.86	24.01
2	0.205,2	45.00	25.00	9.25	23.81	24.01
3	0.205,3	45.00	25.00	9.25	23.80	24.01

results are shown in Table 5. The TFC ranged from 28.865 mg/g to 41.182 mg/g (*m*/*m*) in the extracts from 12 samples. The highest TFC was found in EP extracts from Daping (41.182 mg/g) and the lowest TFC was found in EP extracts from Liping (28.865 mg/g), with a difference of 1.4 times. As a result of the difference in total flavonoids in EP extracts from different areas, it appeared that the TFC in EP was affected by its growth environment.

3.3 The antioxidant activity of EP from 12 regions

The antioxidant activity of EP extracts from 12 origins was determined by DPPH, ABTS, and FRAP evaluation methods, three widely recognized, fast, and accurate methods for measuring antioxidant activity. The results showed that the total flavonoids in EP possessed antioxidant activity, but the antioxidant capacity varied among samples. The results are shown in Fig. 3. The ranges of half inhibitory concentration (IC_{50}) values measured by DPPH, ABTS, and FRAP were 0.348–1.753, 0.361–1.226, and 0.115–0.574 mg/mL, respectively (Table 6). As determined by the three methods, the EP extracts from the Daping area had the strongest antioxidant capacity, while the EP extracts from the Liping area had the weakest antioxidant capacity. Concurrently, the antioxidant activity measured by ABTS and FRAP had the same order of magnitude when compared with DPPH. Therefore, comparing the antioxidant activity of EP extracts, the results of the three assays were consistent. That is, the EP from Daping area had the strongest antioxidant activity, while the EP from Liping area had the weakest antioxidant activity.

The results suggested that the samples with a high content of total flavonoids had strong antioxidant capacity, and the samples with a low content of total flavonoids showed a good dose-activity relationship between the antioxidant capacity of EP extract and the content of total flavonoids. Taken together, these findings provide an experimental basis for future basic research on the pharmacological activities of EP flavonoids.

3.4 The relationship between total flavonoid concentrations, antioxidant activity, and ecological factors at the site of origin

Guizhou is located in the eastern part of the Yunnan-Guizhou Plateau, with a distance of about 595 km from east to west and a distance of about 509 km from north to south. The terrain in the territory is high in the west and low in the east. The province has a total area of 176,167 km^2 and an average elevation of about

Table 5 Ecological information and total flavonoid content in 12 regions

Number	Location	Altitude (m)	Annual mean temperature (°C)	Annual precipitation (mm)	Annual sunshine hours (h)	Annual average relative humidity (%)	Flavonoid contents (mg/g)
S1	Daping	674	16.50	2,290.70	1,031.70	85	41.18 ± 0.76
S2	Gaopo	890	19.20	1,738.90	1,061.30	81	36.99 ± 0.51
S3	Leishan	996	16.10	1,323.50	1,105.00	83	33.46 ± 0.44
S4	Liping	733	17.50	1,604.70	912.60	86	28.87 ± 0.36
S5	Longli	511	14.00	1,452.00	941.00	84	34.59 ± 0.28
S6	Luobang	772	16.60	1,516.50	1,148.70	84	39.88 ± 0.46
S7	Lvyinhu	863	17.00	1,734.60	1,048.10	84	39.71 ± 1.18
S8	Rongjiang	788	17.50	1,604.70	912.60	86	29.97 ± 0.62
S9	Tianzhu	983	14.10	1,567.90	659.90	85	31.54 ± 0.62
S10	Tuanshan	933	15.50	1,576.80	817.80	84	41.00 ± 0.84
S11	Wuzhai	807	15.80	1,080.10	802.00	81	37.08 ± 0.26
S12	Yaobai	941	15.50	1,576.80	817.80	84	34.35 ± 0.78

Table 6 IC_{50} value of DPPH, ABTS, and FRAP antioxidants in EP from different habitats ($n = 3$)

Number	Location	DPPH IC_{50} (mg/mL)	ABTS IC_{50} (mg/mL)	FRAP IC_{50} (mg/mL)
S1	Daping	0.348 ± 0.033	0.361 ± 0.012	0.115 ± 0.025
S2	Gaopo	1.317 ± 0.050	0.877 ± 0.036	0.339 ± 0.019
S3	Leishan	0.621 ± 0.051	0.681 ± 0.054	0.252 ± 0.022
S4	Liping	3.429 ± 0.033	1.226 ± 0.033	0.574 ± 0.059
S5	Longli	0.446 ± 0.027	0.471 ± 0.062	0.151 ± 0.051
S6	Luobang	0.413 ± 0.027	0.384 ± 0.019	0.125 ± 0.067
S7	Lvyinhu	0.619 ± 0.059	0.579 ± 0.034	0.220 ± 0.069
S8	Rongjiang	1.753 ± 0.069	0.981 ± 0.051	0.434 ± 0.062
S9	Tianzhu	1.578 ± 0.027	0.928 ± 0.026	0.441 ± 0.017
S10	Tuanshan	0.394 ± 0.049	0.431 ± 0.066	0.147 ± 0.016
S11	Wuzhai	0.743 ± 0.031	0.705 ± 0.018	0.272 ± 0.062
S12	Yaobai	0.472 ± 0.063	0.481 ± 0.051	0.168 ± 0.033

1,100 m. The landforms can be broadly divided into four basic types: plateau, mountain, hill, and basin. Of these, 92.5% are mountains and hills. It is the only province in China without a plain to support and has a complex climate and environment. EP is mainly distributed in the southeast region of Guizhou Province. According toorts by Han *et al.*[22], the spatial pattern of absolute humidity in Guizhou Province is higher in the east and south, and lower in the west. EP mainly grows in the southeast area, indicating that the ecological environment in this area is suitable for EP growth. The effects of altitude, relative humidity, and annual precipitation on TFC in EP are not clear at present. The relationship between TFC of EP and altitude, temperature, precipitation, sunshine, and humidity was further analyzed. It can provide a theoretical basis for determining the ecological factors that promote the growth of EP.

The flavonoid content of plants can be changed by ecological factors from both abiotic and biotic stresses[23]. Flavonoids are the major secondary metabolites in EP. However, the influence of ecological factors on EP secondary metabolites remains unclear. The ecological factors we considered included altitude, temperature, precipitation, sunshine, and humidity. The relationship between ecological factors and TFC in EP extracts was compared using Pearson's correlation analysis (Table 7). The values obtained for TFC in EP from different origins confirmed that TFC was affected by the growth environment. The analysis showed that the TFC in EP was closely related to the annual sunshine hours and the annual mean temperature. The order of influence was annual sunshine time ($r = -0.841$, $P < 0.01$) > annual mean temperature ($r = -0.723$, $P < 0.01$) > annual precipitation ($r = -0.451$, $P > 0.05$) > altitude ($r = -0.293$, $P > 0.05$) > annual average relative humidity ($r = -0.022$, $P > 0.05$). The results of the effect of ecological factors on antioxidant activity showed that antioxidant activity was closely related to annual sunshine hours and average annual temperature.

We also investigated the relationship between TFC and antioxidant activity (Table 7). The results showed that TFC in EP was significantly negatively correlated with the IC_{50} values obtained using FRAP, DPPH, and ABTS assays. This means that TFC is significantly and positively correlated with antioxidant activity. Indeed, ecological factors appear to strongly affect the antioxidant capacity of EP by modulating flavonoid contents, which have a direct relationship with antioxidant capacity. In our previous study, it was confirmed that EP contains protocatechuic acid, chlorogenic acid, vicenin-2, rutin, hyperosmide isoquercitrin, and quercitrin[11]. Flavonoids have a variety of pharmacological effects, among which it has been confirmed that rutin[24] hypericin[25-27] quercetin[28, 29], isoquercetin[30-32], and other flavonoids have antioxidant effects and antioxidant stress in different environments. Jiang *et al.*[11] found that quercetin was contained in the extract of EP by methanol-water. It was found that the content of quercetin was different by comparing the content of quercetin in the extract of EP from 10 producing areas,

Table 7 Correlation analysis of TFC, anti-oxidant capacity indexes, and the growth environment of EP

Index	Total flavonoids content	FRAP IC_{50}	DPPH IC_{50}	ABTS IC_{50}	Annual sunshine hours	Annual precipitation	Annual mean temperature	Annual mean humidity	Altitude
Total flavonoids content	1.000								
FRAP IC_{50}	−0.807**	1.000							
DPPH IC_{50}	−0.751**	0.946**	1.000						
ABTS IC_{50}	−0.804**	0.991**	0.929**	1.000					
Annual sunshine time	−0.841**	0.847**	0.753**	0.889**	1.000				
Annual precipitation	−0.451	0.126	0.008	0.135	0.257	1.000			
Annual mean temperature	−0.723**	0.398	0.342	0.393	0.547	0.301	1.000		
Annual mean humidity	−0.022	−0.308	−0.243	−0.302	−0.159	0.411	−0.162	1.000	
Altitude	−0.293	0.447	0.484	0.425	0.300	−0.095	0.558	−0.528	1.000

Note: **Significant at $P < 0.01$, *significant at $P < 0.05$.

it was speculated that it might be related to the environment of the producing area. It can be seen that the total flavonoids extracted in this study contain quercetin and other substances with antioxidant properties, which again confirmed our results. The results of the study provide useful information for further application of the medicinal value of EP. However, the specific effects of ecological factors on each flavonoid component of EP, and the causal mechanisms need to be further investigated.

4 Conclusion

In this study, RSM was used to optimize the extraction conditions for UAE of total flavonoids from EP. These conditions include solvent concentration, ultrasonication time, and liquid-solid ratio. Under these optimized conditions, the highest total flavonoid extraction was obtained from EP. An efficient method for the extraction of flavonoids from EP was developed. The TFC and antioxidant activity of EP from 12 regions of Guizhou, China, were determined under optimized conditions. The results showed that different local ecological factors modulated the TFC of EP, resulting in different contents of total flavonoids in EP plants collected from 12 different regions. Both TFC and antioxidant activity were related to ecological factors, especially annual sunshine hours, in 12 regions. The results of the study provide good experimental data for the development and utilization of EP.

Conflicts of interest

The authors declare that there are no conflicts of interest in this work.

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