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Research Article

Application of response surface methodology to optimize the ultrasound-assisted flavonoid-rich extraction of fish mint (*Houttuynia cordata* Thunb.)

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ABSTRACT

Introduction: Fish mint (*Houttuynia cordata* Thunb.) has been widely used in both traditional and modern medicine for a long time. Its flavonoid component has a variety of pharmacological effects that have been demonstrated in previous studies. In this research, we optimized the ultrasound-assisted extraction (UAE) of flavonoid-rich content from *Houttuynia cordata* Thunb. using response surface methodology - central composite design (RSM-CCD). **Methods:** Based on the results of single-factor test , central composite design (CCD) approach-based response surface methodology (RSM) analysis was utilized to evaluate the effects of ethanol concentration, solid-liquid ratio, extraction time, and temperature on the total flavonoid content expressed as rutin equivalents. Flavonoid component from the extract under optimum conditions was then identified by using UPLC-ESI-MS. **Results:** The optimum conditions for obtaining the maximum TFC (53.6321 ± 0.9474 mg RE/g) were found at 80% ethanol concentration, 1/60 g/mL solid-liquid ratio for 38 min at 60 ^{o}C. Using UPLC-ESI-MS, we determined six major flavonoid compounds in the extract: rutin, hyperin, isoquercitrin, quercitrin, afzelin, and quercetin. **Conclusion:** From these results, this study showed that UAE is a fast and efficient technique for flavonoids extraction from the fish mint.

Key words: Fish mint, *Houttuynia cordata* Thunb., flavonoid, UAE, RSM-CCD, UPLC-ESI-MS

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INTRODUCTION

Fish mint (*Houttuynia cordata* Thunb.) is well-known as a detoxification herb that removes toxic heat and promotes drainage of pus [1]. Additionally, it possesses a wide range of pharmacological effects such as antibacterial [2], antiviral [3,4], anti-inflammatory [5], and antioxidant [6] activity as well as the antitumor effect on gastric carcinoma SGC-7901 cells [7] and hepatocellular carcinoma HepG2 cells [8]. Thus, fish mint has been used as a traditional medicine and applied in cosmetics for the treatment of acne and skincare. On top of that, fish mint contributes to the pharmaceutical industry. According to published reports, this herb had many important chemical constituents, including essential oil [9], alkaloid [10], and flavonoid [11], in which flavonoid is the most exciting component. It has been demonstrated that flavonoids in fish mint have plenty of biological activities, for particularly, antibacterial activity against *Pseudomonas aeruginosa, Bacillus subtilis, Escherichia coli,* and *Staphylococcus aureus* [12], anti-free radicals [13], antiviral activity against porcine epidemic diarrhea virus [14], influenza A virus [15], HSV-2 [16] and antitumor effect on Sarcoma-180 cells [17]. By that, flavonoid-rich extract from fish mint becomes a potential ingredient for cosmetics, medicine, and functional food. Therefore, it is necessary to optimize the extraction process to ensure reasonable costs and obtain the flavonoid-rich extract with desired therapeutic effects.

Fish mint extracts can be obtained by many conventional methods such as soaking [18], Soxhlet [13], and reflux [19] extraction. These techniques are simple, easy to perform yet time-consuming and low TFC obtaining. UAE is one of the modern extraction methods that are user-friendly, fast, and efficient with high TFC.

In recent years, RSM has been a popular statistical technique for optimizing multi-factors in manufacturing and doing research [20]. By establishing a mathematical equation, RSM is used for analyzing the interactions between factors affecting one or more responses, known as dependent variables, and figuring out optimum conditions. CCD is one of the common methods to design the experimental procedures of RSM. Compared to other designs, CCD requires fewer experiments but still allow screening of a broad range of parameters as well as the role of each factor.

In this study, we utilized RSM-CCD to optimize the ultrasound-assisted flavonoid-rich extraction of fish mint to provide material extracts for analysis and pharmaceutical manufacturing.

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MATERIALS - METHODS

Materials

Reagents

Ethanol (Merck), sodium nitrite (Xilong Scientific), aluminum chloride hexahydrate (Xilong Scientific), sodium hydroxide (Merck), rutin (Institute of Drug Quality Control Ho Chi Minh City, batch no.: QT152 050417, assay: 88.2%), methanol (HPLC grade, Merck KaGA), acetonitrile (HPLC grade, Merck KaGA), formic acid (HPLC grade, Merck KaGA).

Equipment

Ultrasound bath (Grant, UK), vortex (Stuart, UK), UV-Vis system (Shimadzu, Japan), UPLC-ESI-MS system (Waters, USA).

Object

Fish mint (*Houttuynia cordata* Thunb., Saururaceae).

Sample preparation

Fish mint was bought at Nhan Van market, Linh Trung Ward, Thu Duc City, Ho Chi Minh City, Vietnam and authenticated by Dr. Hoang Viet, Department of Ecology-Evolutionary Biology, University of Science, Vietnam National University Ho Chi Minh City.

Leaves were collected, washed, and dried at 50 ^{o}C for 24 hours (7.19% of moisture content). Then, these dried leaves were ground for 5 min and sieved using a sieve with an opening size of 0.63 mm. The powder was stored in a sealed brown glass bottle placed at room temperature away from sunlight and moisture.

Optimization of the ultrasound-assisted extraction

0.5 g fish mint powder was extracted with ethanol in different concentrations using UAE. The single factor tests were used to determine the preliminary range of the extraction factor that affect the total flavonoid content expressed as rutin equivalents. First, the ethanol concentration (50, 70, 99.7%) was investigated in fixed conditions: 1/40 g/mL sample/solvent ratio, 20 min extraction time, and 30 ^{o}C. The second factor was solid-liquid ratio (1/20, 1/30, 1/40, 1/50, 1/60, 1/70 g/mL) at the same extraction time and temperature using ethanol 70%. Next, the extraction time (20, 40, 60 min) was evaluated using ethanol 70%, 1/50 g/mL solid-liquid ratio at 30 ^{o}C. Finally, the temperature (30, 50, 70 ^{o}C) was investigated using ethanol 70%, 1/50 g/mL solid-liquid ratio for 40 min. For further study, RSM was applied to investigate the interactions between factors and optimize the extraction conditions using Design-Expert software (version 11.1.0.1, Stat-Ease Inc., Minneapolis, MN, USA). We used CCD to design the experimental procedures in RSM. Each independent variable in CCD was coded at five levels: -1 (low), 0 (center), 1 (high), $+\alpha$ and $-\alpha$, where $\alpha = 2^{k/4}$ (k is the number of variables). The range and center point values of these variables were based on the results of single-factor tests.

Determination of total flavonoid content (TFC)

The TFC expressed as rutin equivalents was determined by a spectrophotometric method based on the flavonoid-aluminum complexation method[21]. Calibration curve was constructed by accurately dissolving 0.16, 0.32, 0.48, 0.64, 0.80, and 0.96 mL of rutin stock solution in ethanol 70% (1.0 mg/mL) into 20 mL volumetric flask separately. In each flask, add 6 mL of distilled water and 1 mL solution of $NaNO_2$ (5%, w/v). After 6 min, 1 mL solution of $AlCl_3.6H_2O$ (10%, w/v) was then added. After that, add a 10 mL solution of NaOH (10%, w/v) and adjust with distilled water up to an exact 20 mL. After incubation at room temperature for 15 min, the absorbance was measured at 510 nm with a Shimadzu UV-Vis spectrophotometer (Kyoto, Japan). The amount of $AlCl_3$ was substituted by the same amount of distilled water in the blank.

A similar procedure was employed to prepare a test sample with 0.2 mL of the filtered extract. The TFC was calculated using the formula:

$$TFC = \frac{R \times V \times n}{m \times (100\% - a)} \times 10^{-3}$$

In which:

TFC: total flavonoid content expressed as rutin equivalents (mg RE/g);

R: rutin concentration calculated from calibration curve (μg/mL);

V: volume of extract (mL);

n: dilution factor;

m: weight of sample (g);

a: moisture content of sample (%).

Identification of flavonoids using UPLC-ESI-MS

Test solution preparation: 0.5 g fish mint powder was accurately weighed and extracted at optimum conditions. The crude extract was filtered and evaporated to remove the solvent, then centrifuged with 10 mL

methanol at 5,000 rpm for 10 min. The supernatant was collected and filtered through a 0.45 μm syringe filter.

UPLC-conditions: The chromatography analysis was carried out at 25 ^{o}C using a Waters Acquity UPLC® System and Acquity UPLC® BEH C_{18} column (2.1 × 100 mm, particle size 1.7 μm). The mobile phase consisted of formic acid 0.1% (v/v) (eluent A) and acetonitrile (eluent B) using the gradient procedure, which was as follows: 0-1.25 min: 10% B; 1.25-6.25 min: 10-21% B; 6.25-10 min: 21-31% B. The flow rate was 0.45 mL/min, and the injection volume was set to 1.0 μL.

MS-conditions: The injected samples were ionized with an electrospray ionization (ESI) source in the positive mode (2020 V). The mass range was set to 250-650 *m/z*. The acquired data were processed using MassLynx software (version 4.1, Waters).

Statistical analysis

All experiments were performed in triplicate. The statistical mean and standard deviation (SD) were calculated using Excel 2016 (Microsoft Corporation, Redmond, WA, USA). The results of the RSM were analyzed using Design-Expert software. The analysis of variance (ANOVA) was used to confirm the adequacy of the quadratic model (the p-value of the model and lack of fit should be less than 0.05 and more than 0.05, respectively). The coefficient of determination (R^2) represents the validity and fitness of the model. R^2 values are close to 1, indicating a reasonable adjustment of the model to experimental data. The coefficient of variation (CV) is a measure of the reproducibility of the model.

RESULTS

Optimization of the ultrasound-assisted extraction

Calibration curve for rutin standard at concentrations of 8.0, 16.0, 24.0, 32.0, 40.0, and 48.0 μg/mL was shown in Figure 1. The equation is y = 0.0091x + 0.0093, R^2 = 0.9992 where x is the rutin concentration (μg/mL) and y is the mean absorbance.

The effect of every single factor, including ethanol concentration, liquid-solid ratio, extraction time, and temperature on TFC was evaluated by single factor tests (Figure 2).

Through the series of single-factor experiments, the ANOVA has shown that all four factors significantly affect the TFC ($p < 0.05$). As can be seen from Figure 2A, in a range of 50-99.7% ethanol concentration, the TFC was highest at ethanol 70% and decreased dramatically at ethanol 99.7%. Therefore, 70, 80, and 90% were selected as the low, center, and high levels of ethanol concentration in the RSM study. Six solid-liquid ratios from 1/20 to 1/70 g/mL had positive effects on the extraction of TFC in Figure 2B. The TFC obtained at 1/60 g/mL ratio was higher than at lower levels, but there was no statistical difference compared to the 1/70 g/mL level ($p > 0.05$). Thus, a ratio of 1/60 g/mL was fixed for CCD. Figure 2C shows the effect of extraction time in the range of 20-60 min on TFC. The higher TFC was observed between 20-40 min, so we chose 20, 30, and 40 min as the low, center, and high values in RSM. From Figure 2D, the TFC increased from 30 to 50 ^{o}C before declining to 70 ^{o}C. For RSM, 40, 50, and 60 ^{o}C were selected as the low, center, and high levels.

RSM was used to optimize the extraction procedure. The second-order regression equation shows the relationship between the TFC (Y) and three extraction factors: extraction time (X_1), ethanol concentration (X_2) and temperature (X_3) is as follows:

$$Y = b_0 + b_1 X_1 + b_2 X_2 + b_3 X_3 + b_{12} X_1 X_2 + b_{13} X_1 X_3 + b_{23} X_2 X_3 + b_{11} X_1^2 + b_{22} X_2^2 + b_{33} X_3^2$$

where b_0, b_i, b_{ii}, b_{ij} are the regression coefficients obtained for the intercept, linearity, square, and interaction, respectively.

The central composite design with three factors was applied at five levels including extraction time (13, 20, 30, 40, 47 min), ethanol concentration (63, 70, 80, 90, 97%), and temperature (33, 40, 50, 60, 67 ^{o}C) (Table 1).

The 20 experimental factors are listed in Table 2, which includes six experiments performed at the center point (run 7, 8, 9, 11, 13, 15) to calculate the experimental error. Experimental and predicted response values at different experimental conditions are shown in Table 2.

The ANOVA was performed to evaluate the significance and the fitness of the model as well as the effects of significant individual terms and their interactions on the response (Table 3).

After determining the significance of the model, RSM provided the equation in terms of coded factors using to make predictions about the response as follows:

$$Y = 52 + 0.9896X_1 - 3.53X_2 + 3.05X_3 - 0.4354X_1X_2 + 1.11X_1X_3 + 2.97X_2X_3 - 1.42X_1^2 - 7.18X_2^2 - 0.8343X_3^2$$

The visualization of the significance of the independent variables on the response was shown by contour and 3D surface plots (Figure 3).

The maximum TFC predicted from the RSM was 54.9904 ± 2.0002 mg RE/g for extraction using ethanol 80% with 1/60 g/mL solid-liquid ratio for 38 min at 60 ^{o}C. Six experiments were carried out at the

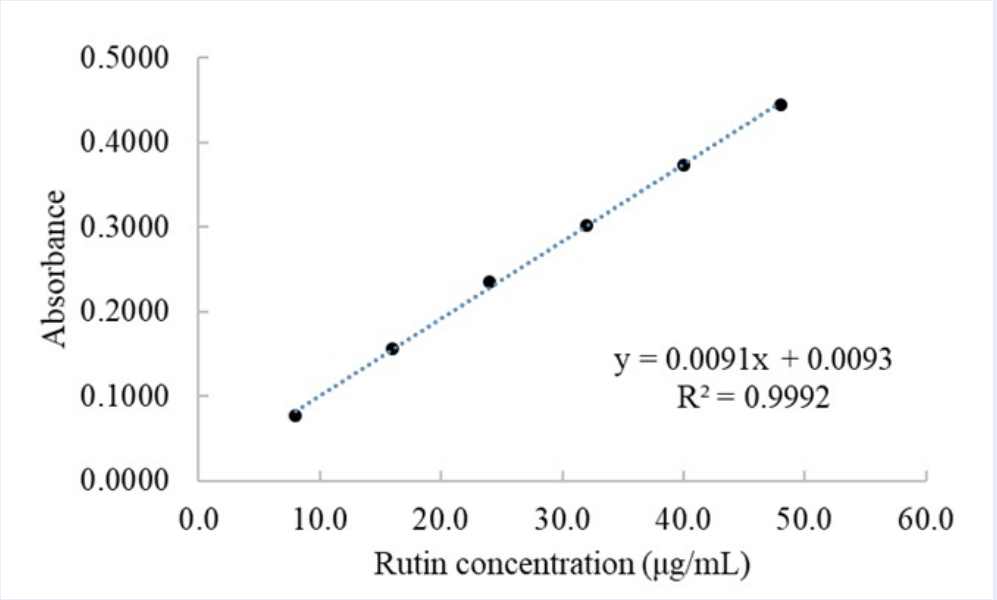

Figure 1: **Rutin calibration curve for the quantification of total flavonoid content**. The results were presented as mean absorbance to each concentration (n = 3, p < 0.05). Regression line equation is y = 0.0091x + 0.0093, R^2 = 0.9992, where x is rutin concentration ranged from 8-48 μg/mL and y is sample absorbance.

Table 1: Variables and factor levels used in the CCD

Actual variables	Coded variables	Factor levels				
		-1.68	-1	0	+1	+1.68
Extraction time (min)	X_1	13	20	30	40	47
Ethanol concentration (%)	X_2	63	70	80	90	97
Temperature (^oC)	X_3	33	40	50	60	67

optimum conditions to validate the accuracy of the applied model equation (Table 4).

Table 4: The actual TFC at optimum conditions

Order	TFC (mg RE/g)
1	51.8405 ± 2.3890
2	54.2227 ± 2.1399
3	53.3648 ± 2.5560
4	54.0680 ± 1.5439
5	53.8890 ± 0.5554
6	54.4077 ± 0.2463
Mean ± SD	53.6321 ± 0.9474
RSD	1.77%

As shown in Table 4, the experimental TFC of optimum extracts was 53.6321 ± 0.9474 mg RE/g (RSD < 2%), which were within 95% confidence interval of predicted values (p < 0.05), indicating that the proposed model is reliable.

Identification of flavonoids using UPLC-ESI-MS

In comparison with theoretical spectral data, the chromatographic analysis identified six signals corresponding to 6 flavonoid compounds, including rutin, hyperin, isoquercitrin, quercitrin, afzelin, and quercetin (Table 5). The chemical structure of these flavonoids was shown in Figure 4.

DISCUSSION

Optimization of the ultrasound-assisted extraction

Design of experiments is a common method to optimize herbal extraction procedures[22]. For fish mint, there were a number of studies on optimization of ex-

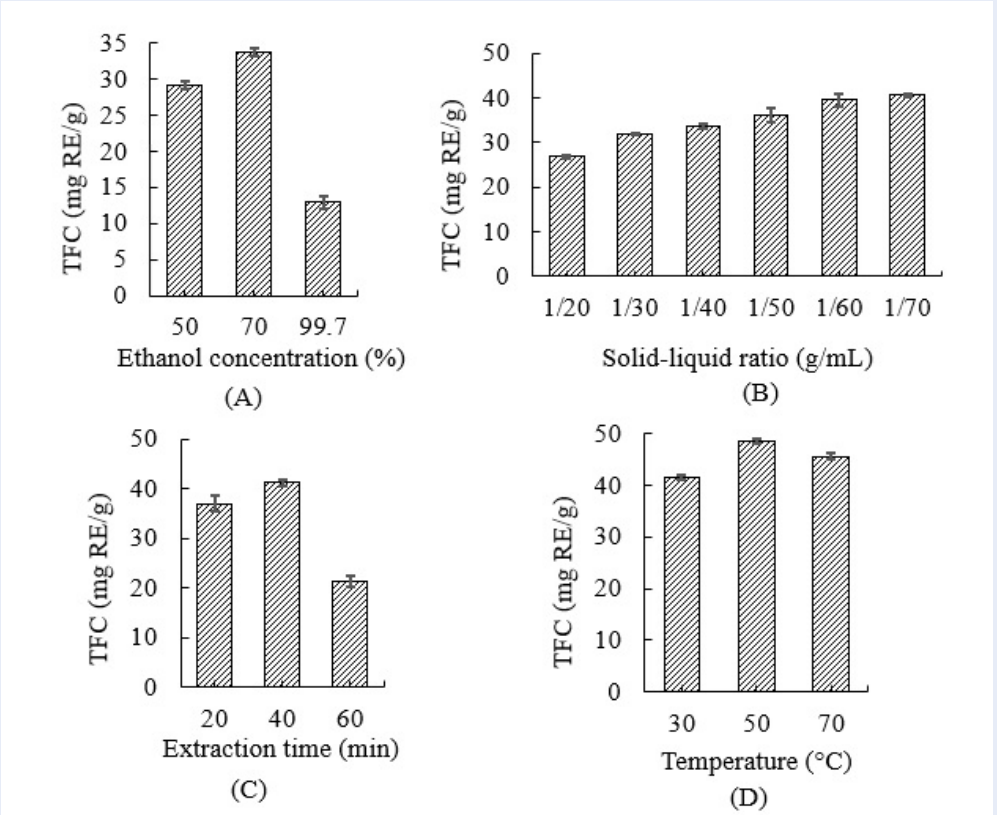

Figure 2: **The effects of ethanol concentration (A), liquid-solid ratio (B), extraction time (C), and temperature (D) on total flavonoids contents (TFC). Values are presented as mean ± standard deviation of three experiments**. Values are significantly different ($p < 0.05$).

traction differing in extraction techniques, design of experiment types as well as response values or dependent variables. Regarding UAE, Kim H. et al.[23] used RSM-CCD aiming to obtain the maximum quercitrin content. While Prommajak T. et al.[24] examined the response values as the maximum total phenolic content and DPPH radical scavenging capacity using RSM-BBD. Another study by Zhang Y. et al.[25] utilized an orthogonal array design to optimize the pressurized liquid extraction for the maximum TFC. In Vietnam, Tuyen N. et al.[19] optimized the reflux extraction to obtain maximum quercetin content using a D-optimal design. However, to the best of our knowledge, so far, there have been no published studies on the utilization of RSM-CCD for optimization of ultrasound-assisted flavonoid-rich extraction from the fish mint.

As can be seen from Table 3, the mathematical model for TFC was significant ($p < 0.0001$), and the lack of fit was insignificant ($p > 0.05$) indicated that the model equation was significant and fit. The high coefficient of determination ($R^2 = 0.9661$) showed the great influences of extraction factors on the TFC. The predicted R^2 of 0.8441 was in reasonable agreement with the adjusted R^2 of 0.9357, indicating a high correlation between predicted and actual values. A low coefficient of variation (CV) of 4.39% denoted that the experimental results were highly reproducible. In general, a process variable can depend on or be depended on by another variable in a set of experiments. Compared to traditional methods, i.e. single-factor tests, RSM has the advantage of evaluating the interaction between couples of factors in the extraction of the responses instead of each factor individually. The application of RSM in the process optimization reduces the number of experimental runs compared to conventional methods, thus saving time, effort, and money. On top of that, the predicted results obtained from RSM are considered to be statistically acceptable[20].

In Figure 3A, the TFC was highest when using ethanol concentration in the range of 75 to 80% for 25-40 min. As increasing ethanol concentration, the TFC reduces

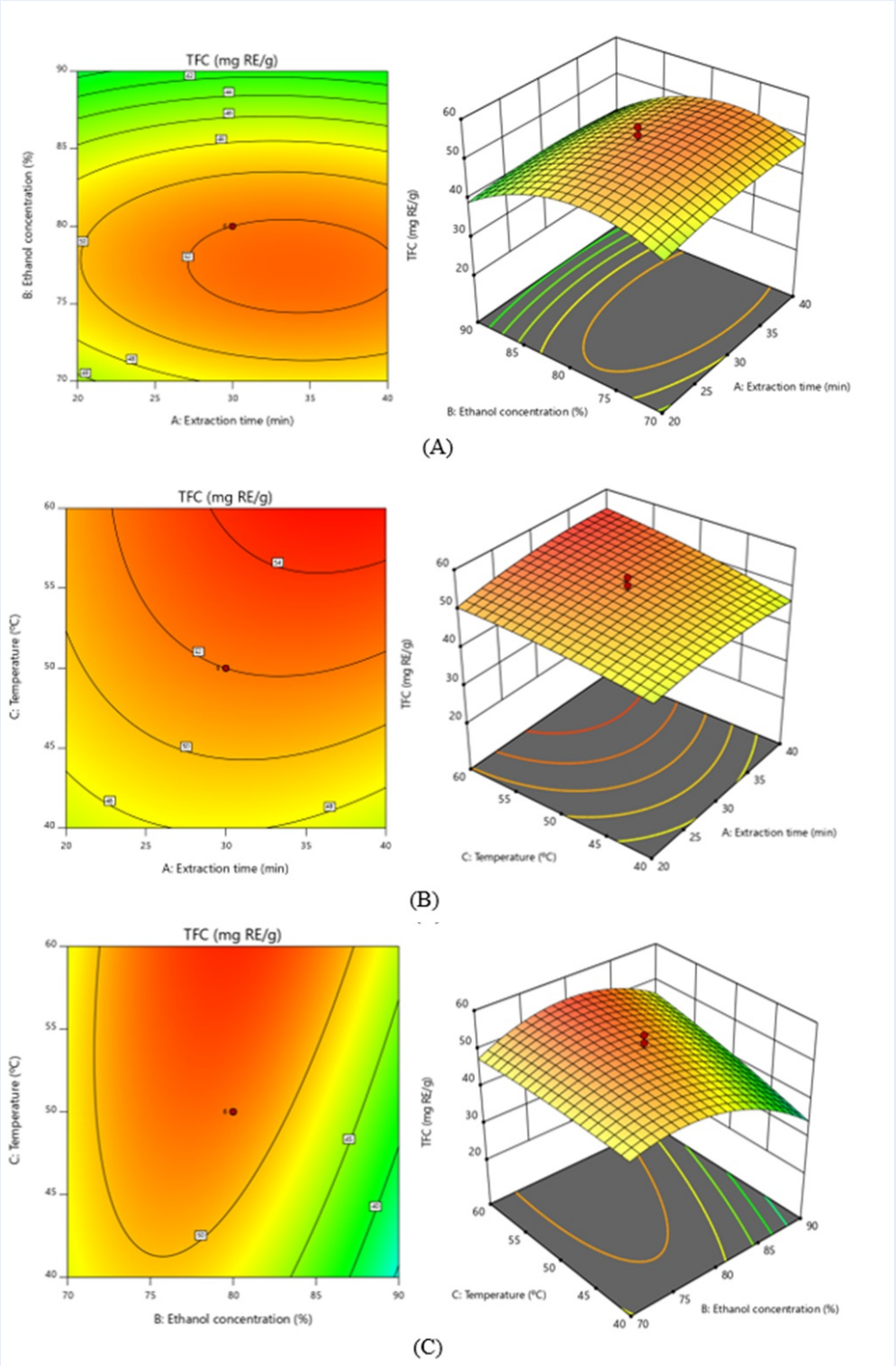

Figure 3: **Contour and 3D surface plots showing the effect of extraction time, ethanol concentration, and extraction temperature on the total flavonoids content (TFC) from *Houttuynia cordata* Thunb.** (**A**) Interactive effects of ethanol concentration and extraction time on TFC; (**B**) interactive effects of extraction temperature and extraction time on TFC; (**C**) interactive effects of ethanol concentration and extraction time on TFC. TFC values are illustrated by colors, increasing from blue, green, yellow, and orange. The red dots at the middle of the plots indicate center points.

Table 2: Experimental design and response values

Run	X_1	X_2	X_3	Actual Y	Predicted Y*
1	30	80	33	44.3912 ± 0.5575	44.5001 ± 2.0002
2	20	90	40	33.2318 ± 0.7038	33.5554 ± 2.0002
3	20	70	40	47.0011 ± 1.6801	45.7083 ± 2.0002
4	40	90	40	32.0760 ± 2.5472	32.5176 ± 2.0002
5	40	70	40	47.0381 ± 0.9837	46.3008 ± 2.0002
6	30	97	50	26.4815 ± 1.5499	25.7628 ± 2.0002
7	30	80	50	55.3440 ± 1.6915	52.0010 ± 2.0002
8	30	80	50	51.5132 ± 2.7300	52.0010 ± 2.0002
9	30	80	50	51.2290 ± 1.4745	52.0010 ± 2.0002
10	47	80	50	49.0927 ± 1.5389	49.6358 ± 2.0002
11	30	80	50	51.0832 ± 1.2053	52.0010 ± 2.0002
12	13	80	50	45.0180 ± 0.9867	46.3618 ± 2.0002
13	30	80	50	53.3437 ± 0.5591	52.0010 ± 2.0002
14	30	63	50	35.0726 ± 0.5750	37.6782 ± 2.0002
15	30	80	50	49.8166 ± 0.2114	52.0010 ± 2.0002
16	20	90	60	43.9997 ± 1.2758	52.0010 ± 2.0002
17	20	70	60	45.3430 ± 1.3536	43.6406 ± 2.0002
18	40	90	60	46.7452 ± 0.5940	46.7037 ± 2.0002
19	40	70	60	50.3786 ± 0.6397	48.7207 ± 2.0002
20	30	80	67	53.0376 ± 2.4128	54.8156 ± 2.0002

*Values are predicted using Design Expert software version 11.1.0.1.

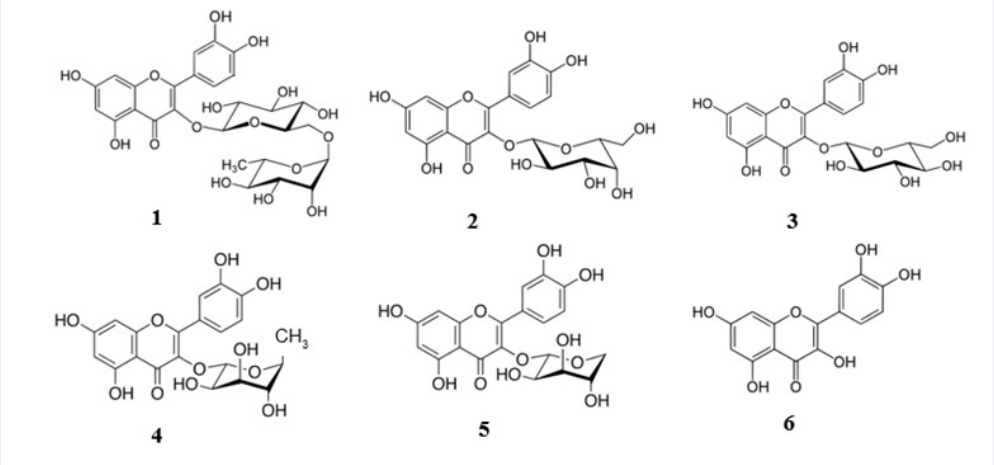

Figure 4: Chemical structure of six flavonoid compounds: rutin (1), hyperin (2), isoquercitrin (3), quercitrin (4), afzelin (5), and quercetin (6)

Table 3: ANOVA for quadratic model and fit statistics

Source	Sum of square	df	Mean of square	F-value	p-value
Model[a]	1141.71	9	126.86	31.71	< 0.0001
X_1-Extraction time	13.37	1	13.37	3.34	0.0974
X_2-Ethanol concentration	169.81	1	169.81	42.44	< 0.0001
X_3-Temperature	127.09	1	127.09	31.77	0.0002
X_1 X_2	1.52	1	1.52	0.38	0.5519
X_1 X_3	9.90	1	9.90	2.47	0.1468
X_2 X_3	70.54	1	70.54	17.63	0.0018
X_1^2	29.09	1	29.09	7.27	0.0224
X_2^2	742.13	1	742.13	185.50	< 0.0001
X_3^2	10.03	1	10.03	2.51	0.1444
Residual	40.01	10	4.00		
Lack of Fit[b]	20.60	5	4.12	1.06	0.4748
Pure Error	19.41	5	3.88		
Cor Total	1181.71	19			
R^2	0.9661				
Adjusted R^2	0.9357				
Predicted R^2	0.8441				
CV%	4.39				

[a]: significant; [b]: not significant

Table 5: MS data of six flavonoids identified in fish mint extract

Order	Retention time (min)	[M + H]+ (m/z)*	[M + H]+ (m/z)**	Name
1	3.675	611.36	611.16	Rutin
2	3.828	465.24	465.10	Hyperin
3	3.964	465.32	465.10	Isoquercitrin
4	4.932	449.23	449.10	Quercitrin
5	5.980	433.15	433.11	Afzelin
6	7.116	303.24	303.05	Quercetin

* Observed values. ** Theoretical value

significantly regardless of extraction time. This trend was reported in extraction processes of various plants such as: *Crinum asiaticum*[26], *Angelica keiskei*[27], and *Celastrus hindsii*[28] . As described in Figure 3B, when the temperature increases, the higher TFC was obtained and less dependent on the extraction time. Theoretically, as rising temperature, both solvent permeability and solubility increase while viscosity decreases, resulting in higher extraction yield. A similar result was reported in *Dendranthema indicum* var. *aromaticum* extraction[29]. However, higher temperatures could cause sensitive flavonoids to be degraded, leading to a decline in the amount of TFC based on the study of Miao Yu et al.[26]. In this study, the designed model indicated that the temperature for high TFC ranges between 50 and 60 ^oC. Similarly, it can be seen in Figure 3 that TFC value reached the highest value at ethanol concentration from 75 to 80% and temperature between 50 and 60 ^oC.

From the RSM, the predicted maximum TFC was 54.9904 $\pm$ 2.0002 mg RE/g for extraction condition including ethanol 80% with 1/60 g/mL solid-liquid ra-

tio for 38 min at 60 ^{o}C.

According to our study, the experimental value of TFC at the optimum conditions was 53.6321 $\pm$ 0.9474 mg RE/g, four times higher than that of the study by Wenguo Cai et al.[6] using ethanol 95% over three extraction times (3 $\times$ 30 min). In comparison with other extraction techniques, Tuyen P. et al.[18] obtained 44.48 $\pm$ 2.77 mg RE/g of the TFC when soaking fish mint in ethanol for three days at room temperature, whereas Chen A. et al.[13] used Soxhlet extraction and just obtained 12 mg RE/g of the TFC. These techniques were all time and solvent consuming but obtained much lower TFC compared to this study. This suggests that UAE is a fast and efficient method to extract flavonoids from the fish mint.

Many factors influence the efficiency of UAE, such as the ultrasonic power, temperature, extraction time, solvent concentration, solid-liquid ratio, and the number of extraction times. Although this is the first study on optimizing the ultrasound-assisted extraction of fish mint to obtain the maximum total flavonoid content expressed as rutin equivalents using RSM-CCD, it was conducted to evaluate the influence of only three factors, including ethanol concentration, extraction time, and temperature. Therefore, the optimization of the extraction process is not yet comprehensive. In addition, it is necessary to investigate the biological activities of flavonoids such as antioxidant, antitumor, and antibacterial activity as other response besides TFC in order to find the optimum conditions under which the obtained extract can be applied for preparation.

Identification of flavonoids using UPLC-ESI-MS

As shown in Table 5, this study preliminarily identified six flavonoid compounds existing in fish mint extract and the elution order of each substance. The data suggest that chromatographic fingerprint of flavonoids from fish mint extract should be established to evaluate the variation of flavonoids in different extraction conditions quantitatively as well as to provide a tool for quality assessment of fish mint extract as the materials and products for cosmetic and pharmaceutical industry.

CONCLUSIONS

This is the first study on optimizing the ultrasound-assisted extraction of fish mint to obtain the maximum total flavonoid content using RSM-CCD. The optimum extraction conditions were found at 80% ethanol concentration, 1/60 g/mL solid-liquid ratio for 38 min at 60 ^{o}C. The experimental TFC of optimum extraction was 53.6321 $\pm$ 0.9474 mg RE/g (RSD < 2%), which were within 95% confidence interval of predicted values. Using UPLC-ESI-MS, six major flavonoids were identified from the extract, including rutin, hyperin, isoquercitrin, quercitrin, afzelin, and quercetin.

LIST OF ABBREVIATIONS

CV: Coefficient of variation (%)

DPPH: 1,1-diphenyl-2-picrylhydrazyl

RE: Rutin equivalent

RSM-CCD: Response surface methodology - Central composite design

RSM-BBD: Response surface methodology - Box–Behnken design

RSD: Relative standard deviation (%)

SD: Standard deviation

TFC: Total flavonoid content

UPLC-ESI-MS: Ultra performance liquid chromatography - Electrospray ionization - Mass spectrometry

COMPETING INTERESTS

The authors declare that we have no competing interests.

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AUTHOR'S CONTRIBUTIONS

All authors contributed in designing and conducting experiments, data analysis and interpretation as well as drafting and revising the manuscript.

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