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Research article

Response surface methodology for aqueous two-phase system extraction: An unprecedented approach for the specific flavonoid-rich extraction of *Houttuynia cordata* Thunb. leaves towards acne treatment

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ABSTRACT

Background: Houttuynia cordata Thunb. has long been widely used as a daily vegetable and traditional medicine. The flavonoid component of *H. cordata* has plenty of pharmacological effects, such as antibacterial, anti-inflammatory, and antioxidant. In this study, we applied the aqueous two-phase system (ATPS) combined with ultrasonic extraction for extracting *H. cordata* leaves.

Methods: We optimized the extraction process to improve the extraction efficiency of the two flavonoids, hyperin and quercitrin, by Surface Method Response - Central Composite Design (RSM-CCD). Next, we investigated the antibacterial ability of *H. cordata* ATPS extract from optimal conditions against two bacterial strains, *Cutibacterium acnes* and *Staphylococcus epidermidis*.

Results: The results showed that using 10% $(NH_4)_2SO_4$ and 35% ethanol for ATPS extraction resulted in the highest hyperin and quercitrin contents. From the RSM-CCD results, the optimal extraction conditions were determined to be ultrasonic extraction at 50 °C for 30 min, giving results consistent with the predicted model and obtaining hyperin and quercitrin contents at 1.5681 ± 0.0114 and 4.6225 ± 0.0327 mg/g, respectively.

Furthermore, ATPS extract has excellent antibacterial activity with a minimum inhibitory concentration (MIC) value of 250 μg/mL on both *C. acnes* and *S. epidermidis*. This MIC is significantly lower than the *H. cordata* ultrasound-assisted (UA) extract, with MICs of 1500.00 and 156.25 μg/mL on *C. acnes* and *S. epidermidis*, respectively. In addition, the results from the disk diffusion

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assay also showed that ATPS extraction has superior internal antibacterial activity with a zone of inhibition diameter at 250 μg/mL of 8.67 ± 1.15 and 5.00 ± 2.00 mm. Meanwhile, those of UA extract on *C. acnes* is 5.67 ± 1.53 mm (at 1500 μg/mL), and on *S. epidermidis* is 1.34 ± 0.58 mm (at 156.25 μg/mL).

Conclusion: To sum up, our research highlights the potential of *H. cordata* ATPS extracts as the starting material for topical preparations for effectively treating acne.

1. Introduction

Acne is a chronic inflammatory disease caused by androgen-induced increased sebum production, altered keratinization, inflammation as well as swelling fortification around pilosebaceous follicles, and proliferation of *Cutibacterium acnes* and *Staphylococcus epidermidis* [1]. Acne causes an unpleasant facial skin condition in teenagers, accounting for over 80% of cases [2]. Although acne does not affect physical health, it dramatically affects psychology, self-esteem, and quality of life. As per European guidelines, the treatment for mild to moderate acne is monotherapy with topical products of retinoids, benzoyl peroxide, or azelaic acid [3]. Combination treatments, such as benzoyl peroxide with adapalene or benzoyl peroxide with clindamycin, are strongly recommended for mild to moderate papulopustular acne treatment. In more severe cases, topical retinoids, specifically adapalene, may be combined with systemic antibiotics [3]. The main problems with conventional acne treatments are antibiotic resistance and local side effects [4]. The trend of choosing products derived from nature is getting more and more attention because the outstanding effects have been proven, such as less occurrence of toxic side effects, significant therapeutic effects for skin diseases, and especially consumer confidence in the safety of natural products [5–7]. According to Statista, natural and organic skincare cosmetics are expected to grow from $9.9 billion in 2021 to $20.4 billion in 2030 [8]. With huge profit potential, the challenge for natural and organic skincare cosmetics manufacturing companies is to create a product of good quality at a low cost that is also environmentally friendly [9].

Houttuynia cordata Thunb. is a perennial herb belonging to the family Saururaceae, widely distributed in Asian regions in general and Vietnam in particular, long used as a vegetable and a traditional remedy [10]. The aerial parts of *H. cordata* have been widely used for diuretics, antibacterial, antifungal and antiviral agents, and treatment of inflammation [11]. *H. cordata* contains several mineral nutrients and the main pharmacologically active components – essential oils and flavonoids [10]. The specific flavonoids are quercetin, rutin, hyperin, quercitrin, and isoquercitrin, which have antineoplastic, antioxidant, antimutagenic, and free radical scavenging capacities [11,12]. Analysis of morphologic and growth traits of a geographic origin revealed that three significant flavonoids were hyperin, quercitrin, and quercetin, in which quercitrin variation was significantly correlated with plant morphology but not with geographic region [13]. Hyperin has been shown to be a crucial active ingredient in multivariate analysis for the quantification and quality control of *H. cordata* [14]. Furthermore, rutin *H. cordata* was proven to effectively prevent UV-induced damages associated with proinflammatory and prooxidative activity and protect cells against apoptosis [15]. *H. cordata* extract has been studied for potential anti-aging and anti-acne properties without attempting to detect immune cell and skin properties [16]. These have become significant advantages in the cosmetics and beauty industry.

Conventional extraction with organic solvent extraction is commonly used in many industries due to its simplicity, lower investment cost, and higher extraction yields [17]. This method adversely affects the preparers and may erode customers' confidence in the product. Green extraction technologies are currently applied to obtain natural compounds intended for multidisciplinary use by utilizing environmentally friendly chemicals, including water of low-concentration alcohol. In the current "green" and sustainable development "green" extraction technology trend, an aqueous two-phase system (ATPS) consisting of a hydrophilic solvent/short-chain alcohol and inorganic salts has been studied and applied in the extraction and purification of natural products from medicinal plants [18]. ATPS integrates partial extraction and concentration in a single step, shortening the isolation and purification process [19]. Compared with conventional liquid-liquid extraction methods, ATPS has an advantage in preserving the target biomolecules because both phases are composed mainly of water [20]. Additionally, this method creates a precise condition for the selective isolation of biomolecules into one of the phases while preserving the essential structure of the biomolecules, resulting in a high yield of extraction and high product purity [19,21]. Along with using cheap, easy-to-find, safe for health, and environmentally friendly solvents, the product obtained by this method has high purity and recovery efficiency, making it easy to successfully industrial scale-up [22,21]. Therefore, utilizing the ATPS extraction method for flavonoid-enriched *H. cordata* extracts is a potential approach in the cosmetics industry that satisfies environmental factors and costs for manufacturers but still meets consumer needs with products of natural origin.

The response surface method (RSM) is a form of experimental design, and the most frequently used is the central composite design (CCD) method. This statistical technique is often applied in multi-factor process optimization by constructing a mathematical expression [23]. Combining RSM-CCD with the ATPS method will help optimize the process, minimizing the experiments needed [22]. However, to our best knowledge, there has yet to be a study on flavonoid-enriched extraction using the APTS method combined with the RSM-CCD technique. Previous studies have reported that hyperin and quercitrin exhibit higher concentrations compared to other flavonoids and are the primary flavonoids responsible for the main antibacterial and anti-inflammatory properties in the *H. cordata* [13,14,24]. The present study aims to utilize the antibacterial effects of H. cordata extracts in formulating acne treatment gel. Thus, we applied the RSM-CCD for the ultrasonic-assisted aqueous liquid two-phase distribution (ATPS) extraction method to optimize the extraction process and improve hyperin and quercitrin content extraction efficiency from *H. cordata* leaves.

2. Materials and methods

2.1. Chemicals

Hyperin, isoquercitrin, quercitrin, quercetin, and rutin were purchased from Sigma-Aldrich, USA. HPLC solvents (Methanol, Acetonitrile, Acid formic, Water) were purchased from Merck KgaA, Germany. Ethanol was provided from Chemsol, China. Ammonium sulfate, potassium dihydrophosphate, sodium biphosphate, potassium carbonate, sodium sulfate and monosodium phosphate were provided from Xilong Scientific, China. Tryptone Soya broth and Tryptone Soya agar were purchased from Alphachem Co., Ltd, Vietnam. Erythromycin was provided by Bioanalyse, Turkey.

2.2. Instrumentals

Ultrasonic water baths (Grant, United Kingdom), Water Bath WNB 14 (Memmert, Germany), Vortex mixer (Stuart, United Kingdom), EBA20S Portable Centrifuge (Hettich, Germany), Analytical balance (Ohaus, United States), HPLC Column Phenomenex C18 Prodigy™ ODS-3100 Å (150 mm × 4.6 mm, 5 μm) (Phenomenex, United States), pH meter (Mettler Toledo, Switzerland), Oven (Memmert, Germany), Shimadzu LC-2030C 3D HPLC System (Shimadzu, Tokyo, Japan), Thermo Scientific Genesys 10S UV–Vis spectrometer (Thermo Scientific, United States), Whitley DG250 Anaerobic Workstations (Whitley, England).

2.3. Objects

H. cordata leaves used in the study were collected at Phu Ha 2 Hamlet, Kien Thanh Commune, Cho Moi District, An Giang Province, Vietnam. We removed old and rotted leaves, washed, drained, and dried them at 50 °C for 36 h in a drying oven (humidity below 10%). Dried *H. cordata* leaves were then ground and sieved through a 0.63 mm sieve to obtain the powder. *H. cordata* leave powder was stored in a tightly closed brown glass bottle at room temperature, away from light and moisture.

2.4. Quantitative analysis of hyperin and quercitrin

Two main flavonoids in *H. cordata* extract, including hyperin and quercitrin, were evaluated using calibration curves that have been previously published [14].

2.5. Phase diagram

The ethanol/salt ATPS system phase diagram developed by turbidity titration was carried out as follows: accurately weighed m_1 (mg) of salt into a 50 mL becher, then added about m_3 (mL) of distilled water to completely dissolve the salt, followed by adding m_2 (mg) of 99.5% ethanol from the burette until the solution became cloudy. The ethanol/salt ATPS systems chosen for investigation are ethanol/$(NH_4)_2SO_4$, ethanol/K_2CO_3, ethanol/KH_2PO_4, ethanol/Na_2HPO_4, ethanol/Na_2SO_4, ethanol/NaH_2PO_4, ethanol/NH_4Cl, ethanol/$(NH_4)_2HPO_4$.

Calculate and draw the phase diagram according to formulas (1) and (2):

$$w_1 = m_1/(m_1 + m_2 + m_3) \quad (1)$$

$$w_2 = m_2/(m_1 + m_2 + m_3) \quad (2)$$

in which:

m_1, m_2, m_3: the weight of salt, ethanol, and water in the system.

w_1 and w_2: the concentrations of salt and ethanol in the system, respectively.

The phase diagrams were illustrated using GraphPad Prism 9 software. The lines divide the phase diagram into two regions: the area below the line is a homogeneous single-phase system without layer separation, and the area above the phase separation line is the region with the phase separation. The layer separates to form two stable phases, with the upper phase being an ethanol-rich phase and the lower phase being a salt-rich phase. The ethanol/salt ATPS system phase diagram enables us to predict proper concentrations of salt and ethanol to form a phase-separated stable system without salt recrystallization.

2.6. Preparation and evaluation of aqueous two-phase system

The ATPS system was prepared by dissolving salt in distilled water in 15 mL falcon and mixed well with ethanol absolute. Vortexed to mix the two phases, then let stand for 15 min to complete the phase separation. The upper and lower phases' volumes were then measured. The final concentrations are demonstrated in Fig. S1.

Calculate the phase ratio R calculated according to formula (3):

$$R = V_t/V_b \quad (3)$$

in which: V_t, V_b: the volumes of the upper and lower phases.

2.7. ATPS extraction procedure

Weighed **a** g of salt into 15 mL falcon, added distilled water to dissolve the salt completely, then added **b** g of 99.5% ethanol. The amount of salt, ethanol, and water is 12 g in total. Weighed **c** g of *H. cordata* leave powder and put it into the falcon. The optimized quantities of a, b, and c are determined in single-factor experiments (Section 2.8). In the next step, we performed ultrasonic extraction for 30 min at 50 °C with a power of 80%. Every 5 min, we removed the vortex, put it in ultrasound again, and then centrifuged at 3000 rpm for 15 min. Siphon the solution above and below the filter through a 0.45 μm filter membrane into a 1.5 mL vial. The aliquots of the two phases are analyzed quantitatively by HPLC, as shown in section 5.4.

Calculate the coefficient of distribution K of each compound by formula (4)

$$K = C_t / C_b \tag{4}$$

in which:

C_t, C_b: the concentrations (mg/mL) of active compounds in the upper and lower phases.

Calculate the recovery rate by formula (5)

$$R_t = \frac{C_t \times V_t \times 100\%}{C_b \times V_b + C_t \times V_t} \tag{5}$$

in which:

R_t: the recovery rate in the upper phase (%)

V_t, V_b: the volumes of the upper phase and lower phase (mL).

Calculate the flavonoid contents by formula (6):

$$C = \frac{X \times V}{1000 \times m \times (1 - M)} \tag{6}$$

in which:

C: the flavonoid content in 1 g *H. cordata* powder (mg/g);

X: the flavonoid concentration in the extract solution (μg/mL);

V: the phase volume (mL);

m: the weight of *H. cordata* powder (g);

M: the moisture of *H. cordata* powder (g).

2.8. Single-factor experiments

We investigated in turn the factors in the ATPS extraction process that affect the flavonoid content, including salt concentration (10%, 12.5%, 15%, 17.5%), alcohol concentration (30%, 35%, 40%), the weight ratio of *H. cordata* powder and ATPS system (1:30, 1:40, 1:50 g/g), extraction time (5, 30, 55 min) and extraction temperature (35, 50, 65 °C). Each experiment was carried out in triplicate; results are presented as mean ± SD.

2.9. RSM-CCD experiment

The experimental design utilizes the response surface method (RSM) with central composite design (CCD) to optimize the extraction process of *H. cordata* with an ultrasonic-assisted ATPS system.

Objective function: The contents of hyperin and quercitrin (mg/g dried *H. cordata* leaves).

Variables: From preliminary survey results, select factors that have an essential influence on the objective function. Each factor will be examined at five levels: Central level (0), high level (+1), low level (−1), +α and −α (where $\alpha = 2^{k/4}$, k is the number of variables).

Number of experiments: $N = 2^k + 2k + 5$. Each experiment was conducted five times at the center to evaluate the error.

Model evaluation: Use Design Expert software (version 13.0) to design experiments and analyze results. Model suitability was determined by analysis of variance (ANOVA). The coefficient of determination (R^2) represents the model's validity; R^2 close to 1 indicates reasonable calibration of the model to the experimental data. According to the relative standard deviation (CV), the coefficient of variation, evaluates the model's reproducibility. The lower the CV, the higher the reproducibility. From there, the software estimates the parameters of the model to predict the influence of variables on the objective function in the form of a regression equation-like expression (7):

$$Y = b_0 + \sum_{i=1}^{k} b_i X_i + \sum_{i=1}^{k} b_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} b_{ij} X_i X_j \tag{7}$$

in which:

Y: the response predicted;

b_0: the coefficient constant;

k: Number of factors; i = 1, 2,3 … k; j = i + 1;

X_i: the independent factor;
b_i: signifies linear coefficient;
b_{ii}: connotes quadratic coefficient;
b_{ij}: the coefficient of interaction.

Finally, from the optimal conditions predicted by the model, the experiments were conducted six times and compared with the predicted results. If the result is within the 95% confidence interval, it shows the model's accuracy.

2.10. Ultra-sound assisted extracted procedure

The ultrasound-assisted (UA) extraction conditions of *H. cordata* leaves followed our previous study [25]. Briefly, *H. cordata* leave powder was weighed and extracted with 80% ethanol with the ratio between powder and solvent 1:60 (g/mL) at 60 °C for 38 min with a power of 80%. The extract was then filtered and concentrated with the low-pressure evaporator.

2.11. Evaluation of antibacterial activity of ATPS and UA extracts at the optimal conditions

2.11.1. Bacterial strains and culture media

This study evaluated two species of gram-positive bacteria (*Cutibacterium acnes* ATCC®11827 and *Staphylococcus epidermidis* ATCC®12228). Gram staining experiments were conducted to determine the morphology—images after staining were captured and recorded. The bacteria were identified as Gram-positive cocci based on the purple-to-blue color observed in their staining. The morphology of C. acnes was observed to be short rod-shaped, while *S. epidermidis* exhibited circular shapes, as shown in Fig. S2. All the bacteria were subcultured on Tryptone Soya Agar (TSA) at 37 °C for 24 h in anaerobic conditions. Bacterial suspensions with turbidity according to the McFarland scale were prepared corresponding to the grade 0.5 ($\sim 10^8$ CFU/mL) in Tryptone Soya broth (TSB). The final inoculum had a value of $\sim 10^5$ CFU/mL.

2.11.2. The minimum inhibitory concentration assay

The broth dilution method was applied to determine the assays' antibacterial activity [26]. Exactly 800 μL of TSB was added to the blank tube with 100 μL of bacterial suspension $\sim 10^5$ CFU/mL. After that, 100 μL of ATPS or UA extract with corresponding concentrations were added to reach a final volume of 1000 μL for each tube. The experimental concentrations of UA extracts and those of ATPS in DMSO ranged from 2500 to 78.125 μg/mL and 1000–7.8125 μg/mL respectively. The positive control of the experiment employed an antibiotic at a tested concentration of 15 μg/mL, while the negative control utilized DMSO at a tested concentration of 10%. Both concentrations were used as substitutes for the experimental concentrations of extracts that was added for investigation. After incubation, the lid was cultured on TSA to observe bacterial development.

2.11.3. Disk diffusion assay

The *C. acnes* strain and *S. epidermidis* strain with $\sim 10^8$ CFU/mL bacterial suspension were spread on TSA using a sterile swab and allowed to dry at room temperature. Sterile 6-mm filter paper discs were impregnated with 10 μL of the prepared extracts. Disks were then allowed to dry at room temperature. Erythromycin 15 μg/mL was placed on the surface of the agar plate as a positive antibiotic control. For the negative control, 10 μL of 10% DMSO was impregnated onto a 6-mm filter paper disc as a substitute for the extracts used in the experimental concentrations. All plates were incubated at 37 °C for 24 h in anaerobic conditions. The diameter of the measured zone minus the diameter of the sterile paper discs equals the zone of inhibition. Each experiment was performed in triplicate, and each strain's zone of inhibition was presented as mean ± standard deviation.

2.12. Statistical analysis

The raw data of HPLC chromatography were examined and exported using LabSolutions software (Version 5.87 SP1; Shimadzu, Japan). ANOVA analyses were performed with Microsoft Office Excel 2016, and RSM-CCD results were analyzed with Design Expert (version 13.0, Stat-Ease Inc., USA). All graphs were illustrated with Graph Pad Prism (version 9.5.0.730, Dotmatics, USA). All experiments were performed in triplicate, and results were presented as mean ± standard deviation.

3. Results

3.1. Aqueous-two phase system for extraction

The phase separation process of ethanol/salt ATPS is due to the "salting-out" phenomenon and competition of salt and ethanol for water particles [27]. The difference in affinity between ion-water and ethanol-water, along with interactions between ethanol molecules, leads to the separation of ethanol from the salt solution or crystallization of the salt [28]. The flavonoids at various concentrations dispersed in the two phases based on the difference in their affinity against water and ethanol molecules. The salt solubility in ATPS results are described in Fig. S1 and Table S1. The results showed that all the salts have great solubility; however, except for K_2CO_3 and $(NH_4)_2SO_4$, the remains are unstable and prone to crystallization in ethanol-water co-solvency, which is inappropriate for ATPS extraction. Only K_2CO_3 and $(NH_4)_2SO_4$ salts exhibit reasonable and stable phase separation when adding ethanol. Thus, the ethanol/$(NH_4)_2SO_4$ and ethanol/K_2CO_3 systems were chosen to investigate the contents of flavonoids after the UA-ATPS extraction.

3.2. Optimization of ATPS conditions to obtain flavonoid-rich extract using single-factor experiments

After ATPS extraction, the extracts in two phases were collected, and the pH was measured. The results show that the alcohol-rich phase (upper phase) and the salt-rich phase (lower phase) had a pH of ~ 5 and 4, respectively. The extract was then filtered through a 0.45 μm filter and quantified the flavonoid contents by HPLC. All five specific flavonoids, including rutin, hyperin, isoquercitrin, quercitrin, and quercetin, were detected in the alcohol-rich phase, while in the salt-rich phase, they were almost not detected. When adjusting the pH of the salt-rich phase to approximately 5, flavonoids were detected with larger peak areas because they transformed into their free forms. This pH of 5 is also compatible with the solvent for RP-HPLC analysis condition, resulting in the precise retention time and peak areas [29].

The hyperin and quercitrin contents in the two phases, the distribution coefficient, and the recovery rate of the alcohol-rich phase (upper phase) are shown in Table 1. Both ethanol/salt ATPS systems have high recovery rates in the upper phase. In ethanol/K_2CO_3 ATPS system, the solution of K_2CO_3 is slightly alkaline; thus, flavonoids undergo deprotonation and increasing ionic strength, decreasing the solubility of flavonoids in solvent and lower extraction yield. Meanwhile, when using the ethanol/$(NH_4)_2SO_4$ ATPS system, hyperin and quercitrin have higher concentrations in the alcohol-rich phase because, in an acidic environment, flavonoids are protonated, increasing the interaction of flavonoid molecules and the hydrophobic surface of the solvent [30].

The contents of hyperin and quercitrin in the extracts by the ATPS system using ethanol/$(NH_4)_2SO_4$, ethanol/K_2CO_3 compared with solely UA method extracting by ethanol 70% and 80% are shown in Table S2. The results showed that the flavonoids had the highest contents using ATPS with ethanol 35%/$(NH_4)_2SO_4$.

The effects of ethanol concentration, salt concentration, powder-solvent ratio, extraction time, and temperature were determined using single-factor experiments, and the results have been demonstrated in Fig. 1. As illustrated in Fig. 1A, the optimal concentrations of hyperin and quercetin were obtained with statistically significant differences ($p < 0.01$) when utilizing 35% ethanol. An ethanol concentration of 35% was maintained for further experiments. The $(NH_4)_2SO_4$ concentration (%, w/w) ranging from 10% to 17.5% were surveyed. The data revealed that the contents of hyperin and quercitrin reached the highest values at a salt concentration of 10%, with $p < 0.0001$ (Fig. 1B). The quercitrin content is notably affected by solvent and material ratio, while such variations do not significantly impact the hyperin content, as shown in Fig. 1C. Consequently, we maintained a fixed ratio of 40:1 for solvents and material during the subsequent investigations to mitigate solvent consumption. The extraction time factor was examined over 5–55 min, and the highest contents of hyperin and quercitrin were achieved at 30 min (Fig. 1D). From Fig. 1E, an extraction temperature of 50 °C was identified as optimal for hyperin and quercitrin content, with a statistically significant difference ($p < 0.0001$).

3.3. RSM-CCD for optimization of extraction

3.3.1. Optimization of extraction conditions with the ATPS system using the RSM-CCD model

Results from single-factor experiments showed that ethanol and salt concentrations in the ATPS system influence the phase separation. Furthermore, the extraction time and temperature significantly affect the concentration of hyperin and quercitrin, while the ratio of solvent to material showed a negligible effect on the extraction efficiency. Consequently, based on single-factor experiment results (Section 3.2), 35% ethanol, $(NH_4)_2SO_4$ 10%, and the ratio 40:1 was chosen as the constant parameters for ATPS systems, and the RSM-CCD was designed to optimize time and temperature factor for the ATPS extraction.

The CCD model was designed for experiments varying in extraction temperature (X_1) and extraction time (X_2) with five different experimental levels described in Table S3. After determining the survey level for each factor, we constructed a matrix and assessed extraction efficiency. The respective components and outcomes of each experiment are described in Table 2. The extraction temperature (X_1) variable was examined from 35 °C to 65 °C, and the extraction temperature variable (X_2) was examined from 5 to 55 min. Response variables of the system consisted of hyperin and quercitrin content. We conducted 13 experiments, which had five as replication of the central points (50 °C in 30 min) to obtain actual results for the RSM-CCD prediction.

3.3.2. Validation of RSM-CCD model

The ANOVA and model fit analysis on hyperin and quercitrin content results are presented in Tables 3 and 4, respectively. The correlation coefficient of R^2 of the hyperin content model was 91.91% and 93.6% for the quercitrin content model, which are good similarities between the predicted and experimental results. The values adjusted R^2 are just slightly lower than R^2, which confirmed that the models are consistent with the experimental data. The models' lack of fit is higher than 0.05 (0.2564 and 0.1268 > 0.05), showing that the regression model of this study is a suitable data model. Moreover, in both hyperin and quercitrin content models, the

Table 1
Contents (mg/g) of hyperin and quercitrin, distribution coefficient K, and recovery rate Rt (%) in the upper phase using the ethanol/$(NH_4)_2SO_4$, and ethanol/K_2CO_3 ATPS system.

Compounds		Hyperin			Quercitrin		
ATPS system	Phase	Content (mg/g)	K	Rt (%)	Content (mg/g)	K	Rt (%)
Ethanol/K_2CO_3	Upper phase	0.1340	–	100	6.6737	75.88	96.52
	Lower phase	–			0.2404		
Ethanol/$(NH_4)_2SO_4$	Upper phase	1.2960	42.79	98.77	11.0537	40.51	98.70
	Lower phase	0.0161			0.1451		

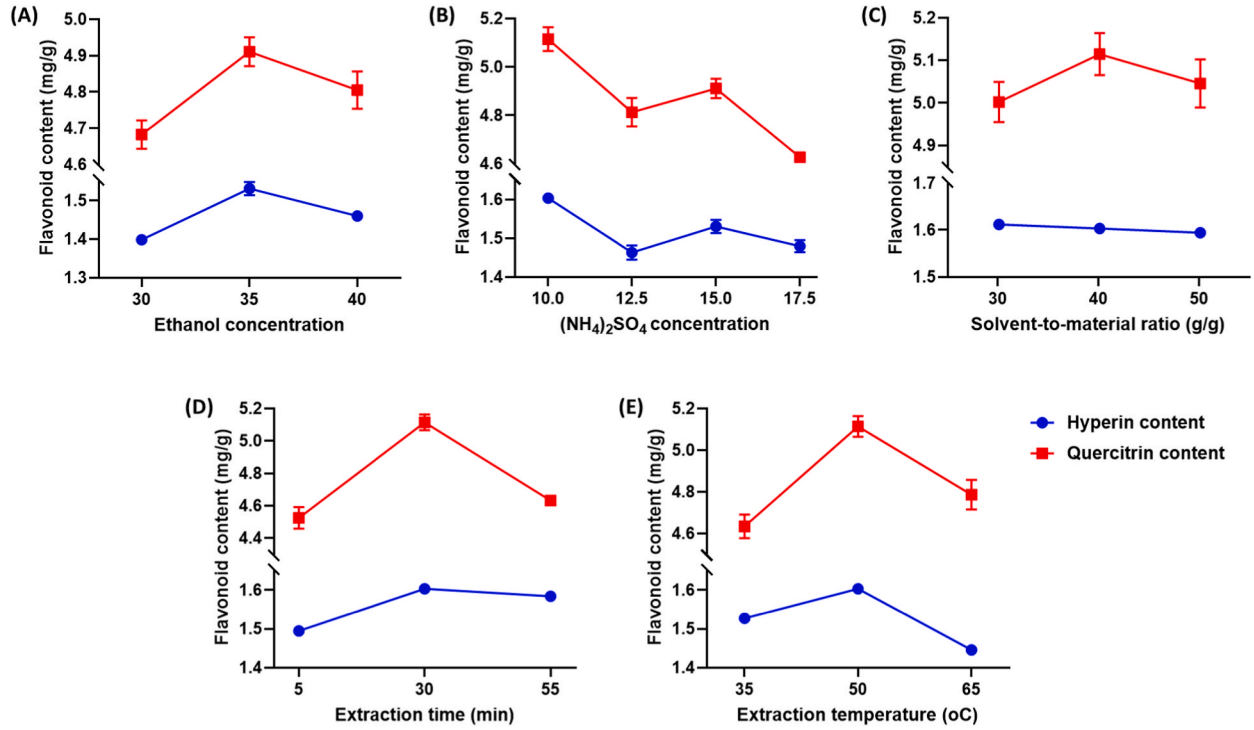

Fig. 1. Effect of ethanol concentration (%, g/g) (A), salt concentration (%, g/g) (B), the ratio between solvent and powder (g/g) (C), extraction time (D), and extraction temperature (°C) (E) on the contents of hyperin and quercitrin.

Table 2
Experimental and predicted hyperin and quercitrin content results.

No.	X_1	X_2	Y_1* (mg/g)	Y_1** (mg/g)	Y_2* (mg/g)	Y_2** (mg/g)
1	60.61	12.32	1.5573	1.5560	4.6266	4.6275
2	50.00	55.00	1.5643	1.5477	4.4115	4.4054
3	35.00	30.00	1.5107	1.5166	4.4701	4.4721
4	50.00	30.00	1.5812	1.5654	4.6146	4.6098
5	60.61	47.68	1.5175	1.5180	4.3721	4.4099
6	50.00	30.00	1.5724	1.5654	4.6275	4.6098
7	39.39	47.68	1.5658	1.5668	4.5017	4.5108
8	50.00	30.00	1.5694	1.5654	4.6028	4.6098
9	50.00	30.00	1.5602	1.5654	4.6082	4.6098
10	39.39	12.32	1.4838	1.4723	4.4424	4.3718
11	50.00	5.00	1.5082	1.5077	4.4525	4.4610
12	65.00	30.00	1.5639	1.5411	4.6193	4.5816
13	50.00	30.00	1.5575	1.5654	4.6594	4.6098

Notice: *Experimental results. **Predicted results using Design Expert 13.0.

p-values of the quadratic terms for the factors X_1 and X_2 are lower than the significance level, demonstrating that these factors significantly affect the flavonoid contents in the extracts. The interaction between two pairs of factors X_1 - X_2 also significantly affects each other with a p-value higher than 0.05. Overall, the temperature and time extraction have a synergetic effect on hyperin and quercitrin contents. Two factors also positively correlate with the concentrations of these two flavonoids. However, higher temperature and prolonged time extraction would lower the flavonoid contents.

The RSM-CCD also provided the equation of target function Y as follows:

$$Y_1 = 1.57 + 0.0152X_1 + 0.0126X_2 - 0.0304X_1X_2 - 0.0174X_1^2 - 0.0168X_2^2$$

$$Y_2 = 4.62 - 0.0316X_1 + 0.0332X_2 - 0.0785X_1X_2 - 0.0959X_1^2 - 0.0396X_2^2$$

in which:

X_1: the extraction temperature;

X_2: the extraction time;

Table 3
Analysis of variance for quadratic model and fit statistics of hyperin content.

Source	df	Sum of square	Mean of square	F-value	p-value
Model	0.0104	5	0.0021	15.92	0.0011
X_1-Extraction temperature	0.0013	1	0.0013	9.63	0.0172
X_2-Extraction time	0.0018	1	0.0018	14.11	0.0071
X_1X_2	0.0037	1	0.0037	28.33	0.0011
X_1^2	0.0020	1	0.0020	15.07	0.0060
X_2^2	0.0021	1	0.0021	16.02	0.0052
Residual	0.0009	7	0.0001		
Lack of Fit	0.0005	3	0.0002	2.00	0.2564
Pure Error	0.0004	4	0.0001		
Cor Total	0.0113	12			
R^2	0.9191				
Adjusted R^2	0.8614				
Predicted R^2	0.6045				
CV%	0.7395				
Adeq Precision	11.8875				

Table 4
Analysis of variance for quadratic model and fit statistics of quercitrin content.

Source	df	Sum of square	Mean of square	F-value	p-value
Model	0.1106	5	0.0221	20.72	0.0005
X_1-Extraction temperature	0.0088	1	0.0088	8.26	0.0238
X_2-Extraction time	0.0080	1	0.0080	7.50	0.0290
X_1X_2	0.0246	1	0.0246	23.06	0.0020
X_1^2	0.0109	1	0.0109	10.20	0.0152
X_2^2	0.0640	1	0.0640	59.93	0.0001
Residual	0.0075	7	0.0011		
Lack of Fit	0.0054	3	0.0018	3.54	0.1268
Pure Error	0.0020	4	0.0005		
Cor Total	0.1181	12			
R^2	0.9367				
Adjusted R^2	0.8915				
Predicted R^2	0.6460				
CV%	0.7199				
Adeq Precision	11.0096				

Y_1: hyperin content;
Y_2: quercitrin content.

From the theoretical and experimental model, the optimal conditions for ATPS extraction of *H. cordata* were at 50 °C in 30 min with 35% ethanol, 10% of $(NH_4)_2SO_4$, and the solvent-to-material ratio of 40:1. The predicted results of hyperin and quercitrin contents are 1.5681 ± 0.0114 and 4.6225 ± 0.0327 mg/g, respectively. The results of hyperin and quercitrin contents in six ATPS extracts at the optimal conditions are shown in Table 5. The actual results for samples extracted under optimal conditions are not significantly different from those predicted by the software. The hyperin and quercitrin contents from the ATPS extracts were 1.5709 ± 0.0119 and 4.6375 ± 0.0138 mg/g, respectively.

3.3.3. Comparison of different extraction methods at the optimal conditions based on the contents of hyperin and quercitrin

The contents of hyperin and quercitrin by UA extraction with 80% ethanol under optimal conditions are 1.2889 ± 0.0062 mg/g and 3.9387 ± 0.03 mg/g, respectively as described in Table 6. Compared to the UA extraction method, the extraction with the ATPS yields 1.22 and 1.18 times higher hyperin and quercitrin contents, which are 1.5709 ± 0.0119 and 4.6375 ± 0.0138 mg/g.

Table 5
The hyperin and quercitrin contents at optimal conditions for ATPS extraction.

Experiment number	Hyperin content (mg/g)	Quercitrin content (mg/g)
1	1.5770	4.6370
2	1.5553	4.6192
3	1.5788	4.6243
4	1.5869	4.6530
5	1.5647	4.6400
6	1.5627	4.6515
Mean	1.5709	4.6375
% RSD	0.76	0.30

3.4. Evaluation of antibacterial activity of ATPS and UEA extracts at the optimal conditions

The MIC values and zone of inhibition diameters obtained at the highest concentration are presented in Table 7. *H. cordata* ATPS extracts showed the antibacterial activity with a MIC value of 250 μg/mL against both *C. acnes* and *S. epidermidis*. While *H. cordata* ATPS extract exhibited superior antibacterial activity on *C. acnes*, with a 6-fold lower MIC value than this of UA extract, UA extract only had a slightly more potent inhibitory against *S. epidermidis*, with a 1.6-fold decrease in MIC values (156.25 μg/mL) compared with ATPS extract. These detailed results are demonstrated in Tables S4 and S5.

The results in Fig. 2 indicated that the diameter of the inhibition zone increases proportionally with the concentration. As shown in Fig. 2C, for the *C. acnes* strain, the diameter of the inhibition zone at a concentration of 1000 μg/mL for ATPS extract (22.0 ± 1.00 mm) was lower than that of erythromycin at 15 μg/mL (29.67 ± 1.15 mm) by only 4.5%, and still superior to the UA extract at 2500 μg/mL (10.67 ± 0.58 mm). Regarding the *S. epidermidis* strain, the UA extract at the highest concentration of 2500 μg/mL yielded a diameter of 31.33 ± 2.52 mm in Fig. 2E, which was twice the diameter yielded by the ATPS method at the highest concentration of 1000 μg/mL, with a diameter of 16.67 ± 1.16 mm in Fig. 2G. All detailed results are illustrated in Table S6.

4. Discussion

The study has successfully utilized the ATPS method in combination with RSM-CCD to obtain a flavonoid-rich *H. cordata* extract. The model has provided optimal conditions for ATPS extraction, which includes 35% ethanol, 10% $(NH_4)_2SO_4$, and a solvent-to-material ratio of 40:1 at 50 °C for 30 min. The correlation coefficients R^2 (hyperin model with 91.91% and quercitrin model with 93.6%) and lack of fit (>0.05) of both hyperin and quercitrin indicate that the two models are suitable and similar. The model predicted the relative accuracy of the specific flavonoid contents at 1.5681 ± 0.0114 for hyperin and 4.6225 ± 0.0327 mg/g for quercitrin, compared to the actual contents of hyperin and quercitrin at 1.5709 ± 0.0119 and 4.6375 ± 0.0138 mg/g, respectively. Furthermore, in a comparison between ATPS extraction methods and other methods such as hot soaking, ultrasound-assisted extraction, and pressurized lipid, the ATPS method achieved higher amounts of hyperin and quercitrin, particularly with quercitrin enrichment being 2–3 times higher than those of the traditional methods [31]. To sum up, our study reveals the capacity of the ATPS method accompanied by the RSM-CCD model to extract and enrich specific flavonoids in low ethanol better than conventional extraction methods, even with organic solvents.

H. cordata has long been used as a daily vegetable and traditional herb in some Asian countries. In previous studies, depending on the research purpose, *H. cordata* leaves are mainly extracted using benign and safe solvents such as water and organic solvents such as ethanol, chloroform, methanol, and *n*-hexane [32]. Flavonoid-rich extraction of *H. cordata* commonly uses ethanol-water for extraction with high concentrations of ethanol ranging from 50% to 96% by maceration, percolation, or a more advanced ultrasound-assisted extraction method [33,34]. The average hyperin and quercitrin content in *H. cordata* were 4.313 mg/g and 5.738 mg/g, respectively [33]. According to Kim et al., the study established RSM-CCD's optimal extract condition with 95% methanol at 80 °C in 34 min by RSM-CCD [34]. The optimal condition obtained a 1.2% amount of quercitrin, higher than conventional ethanol-water extraction. Although utilizing organic solvents results in high extraction yields of bioactive compounds, this approach has harmful effects on the preparers and may undermine consumers' trust in the product. Besides, this can make it challenging for scaling up towards industrial applications [35]. Meanwhile, the ATPS method is a potential solution for utilizing environmentally friendly chemicals such as low-concentration alcohol, aligning with the "green" extraction technology trend while still providing higher extraction yields than conventional methods. Moreover, the previous study by Zhang et al. showed that the ATPS extraction method could potentially obtain flavonoid compounds with higher purity and more than 80% higher than the ultrasound-assisted extraction method [36]. The combination of ATPS extraction and RSM-CCD has demonstrated high reliability and reduced time and cost.

The study was also conducted on the antibacterial activities of *H. cordata* extracts using the ATPS extraction method against the *C. acnes* and *S. epidermidis* strains, which are responsible for human acne vulgaris [37]. The MIC values of ATPS *H. cordata* extract against *C. acnes* and *S. epidermidis* were 250 μg/mL for both strains, indicating its potential as an outstanding antibacterial agent for *C. acnes*. These findings highlight the potential of *H. cordata* as a valuable source of raw materials for the pharmaceutical and cosmetics industry.

Research on *H. cordata* is still limited, with the primary focus being on its antiviral and anti-aging properties [38,39]. Most antibacterial activity studies have been performed on *Bacillus* sp., *E. coli*, and *Staphylococcus aureus* [40,41]. Notably, in 2022, a study by Phosri et al. (2022) investigated the antibacterial activity of *H. cordata* using aqueous and ethanol extraction methods [16]. The MIC values against the *C. acnes* strain for the aqueous and ethanol extracts were 5.77 mg/mL and 2.47 mg/mL, respectively, significantly higher than the values obtained in the current study. This difference further underscores the potential of the ATPS extraction method for specific flavonoid enrichment in *H. cordata* for further application in cosmetics. It is noteworthy that this is the first combined model of the ATPS method and RSM-CCD for specific flavonoid-rich extract.

5. Conclusions

We successfully combined the ATPS extraction method and experimental design based on RSM-CCD to optimize the extraction process of specific flavonoid-rich extract compared to other traditional methods. The obtained extract has antibacterial activity against *C. acnes* and *S. epidermidis* with MIC values of 0.25 mg/mL, which has the potential for further application in topical preparations.

Table 6
The hyperin and quercitrin contents of *H. cordata* ATPS and UA extracts from the optimal conditions

Flavonoid content (mg/g)	UA extract	ATPS extract
Hyperin	1.2889 ± 0.0062	1.5709 ± 0.0119
Quercitrin	3.9387 ± 0.0300	4.6375 ± 0.0138

Table 7
Antibacterial test results on ATPS and UA extracts against *C. acnes* and *S. epidermidis*.

	C. acnes		S. epidermidis	
	MIC (μg/mL)	Zone of inhibition (mm)	MIC (μg/mL)	Zone of inhibition (mm)
ATPS extract	250.00	22.00 ± 1.00	250.00	16.67 ± 1.16
UA extract	1500.00	10.67 ± 0.58	156.25	31.33 ± 2.52
Erythromycin (15 μg/mL)	–	29.67 ± 1.15	–	20.33 ± 1.53

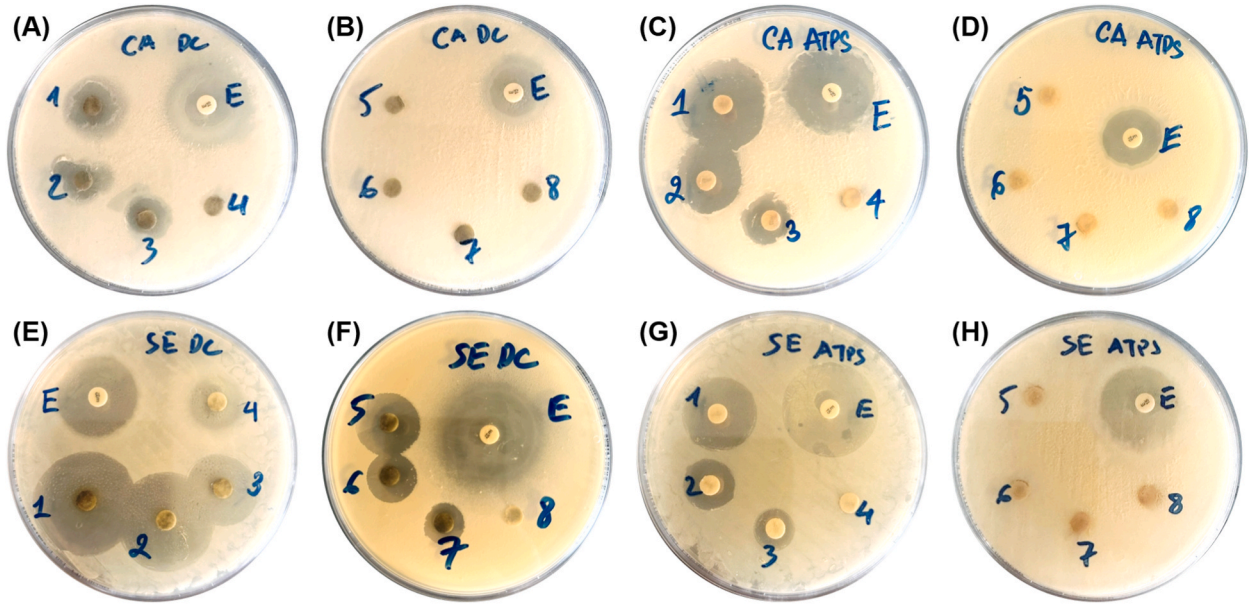

Fig. 2. Zone of inhibition diameters of *H. cordata* extracts against *C. acnes* are denoted as (A)–(D) and *S. epidermidis* as (E)–(H). While ATPS extracts (C), (D), (G), and (H) employed a 2-fold dilution with concentrations ranging from 1000 to 7.8125 μg/mL, labeled as 1–8; UA extracts (A), (B), (E), and (F) were a 2-fold dilution series with concentrations ranging from 2500 to 78.125 μg/mL, also labeled as 1–8, respectively. Erythromycin at 15 μg/mL was used as the positive control, denoted as E.

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Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Minh Hien Nguyen: Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Lan Thi Nguyen:** Writing – original draft, Methodology, Investigation, Formal analysis. **Thien Han Nguyen Le:** Writing – original draft, Visualization, Investigation. **Trong Nghia Ngoc Chau:** Writing – original draft, Methodology, Investigation. **Yen Nhi Thi Nguyen:** Writing – original draft, Visualization, Investigation. **Tan Dat Ha:** Writing – original draft, Methodology, Investigation. **Phuoc Thuan Tran Nguyen:** Writing – original draft, Methodology, Investigation. **Thien Bao Chu:** Writing – original draft, Investigation. **Chi Hieu Tran:** Writing – original draft,

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviation

ATPS Aqueous two-phase system
C. acnes Cutibacterium acnes
H. cordata *Houttuynia cordata* Thunb
MIC Minimum inhibitory concentration
TSA Tryptone Soya agar
TSB Tryptone Soya broth
RSM-CCD Surface Method Response - Central Composite Design
S. epidermidis Staphylococcus epidermidis
UA Ultrasound-assisted

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e25245.

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