DOI: 10.58490/ctump.2025i9TA.3800

OPTIMIZATION OF THE EXTRACTION PROCESS OF POLYPHENOLS FROM *EHRETIA ASPERULA* LEAVES IN CAN THO

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Received: 22/3/2025

Reviewed: 05/5/2025

Accepted: 25/6/2025

ABSTRACT

Background: *Ehretia asperula is a medicinal plant rich in polyphenols, which have significant antioxidant properties. Optimizing the extraction process is essential to enhance polyphenols yield and maximize its potential applications in pharmaceuticals.* ***Objectives:*** *To optimize the extraction conditions of polyphenols from Ehretia asperula leaves using the Box-Behnken design.* ***Materials and methods:*** *The optimization was performed using Design Expert 13.0, evaluating three independent variables: temperature (60°C, 65°C, 70°C), solvent-to-material ratio (1/30, 1/35, 1/40), and ethanol concentration (60%, 70%, 80%). Total polyphenol content was determined using the Folin-Ciocalteu method.* ***Results:*** *The optimal extraction conditions were identified as an ethanol concentration of 67.28%, a temperature of 68.91°C, and a solvent-to-material ratio of 1/35.1557 (w/v), yielding 96.442 mg GAE/g of extract. Experimental validation showed a polyphenol content of 89.593 mg GAE/g, confirming the model's reliability. Additionally, the study determined that five extraction cycles were necessary to achieve maximum polyphenols recovery.* ***Conclusions:*** *The optimized extraction process significantly enhances the yield of polyphenols from Ehretia asperula, providing a reliable method for future pharmaceutical and nutraceutical applications.*

Keywords: *Ehretia asperula, polyphenols extraction, optimization, Box-Behnken design.*

I. INTRODUCTION

Ehretia asperula (*E. asperula*) is a medicinal plant widely used in traditional medicine due to its high polyphenol content, which exhibits strong antioxidant, anti-inflammatory, and antimicrobial properties, making it valuable for pharmaceutical and nutraceutical applications [1]. Polyphenols have been shown to protect against oxidative stress-related diseases, including cardiovascular disorders, neurodegenerative diseases, and certain cancers [2], [3]. Their extraction efficiency depends on factors such as solvent concentration, temperature, and solvent-to-material ratio, requiring optimization to maximize yield while preserving bioactivity. Ethanol-water mixtures are commonly used, but variations in extraction conditions can significantly impact efficiency [4], [5]. Response surface methodology (RSM), particularly the Box-Behnken design, has proven effective in optimizing bioactive compound extraction from medicinal plants [6], [7]. This study aims to optimize polyphenol extraction from *E. asperula* leaves using the Box-Behnken design, providing a scientific foundation for large-scale production of polyphenol-rich extracts for pharmaceutical applications.

II. MATERIALS AND METHODS

2.1. Materials

The *E. asperula* leaves were collected from Can Tho City. The genetic sequencing of the research sample was conducted at the Department of Genetics and Breeding, Faculty of Agriculture, Can Tho University. The sample was recorded under the identification code Sample_26012024 and compared with the reference *E. asperula* sample (ID: OQ139468.1).

2.2. Methods

- Preparation of herbal powder and analysis of chemical components:

Fresh *Ehretia asperula* leaves were washed, dried at 50ºC until moisture content was <13%, then ground and sieved (1400/355). The obtained powder was stored in sealed containers at room temperature, protected from light and humidity, for subsequent experiments. Qualitative phytochemical analysis was performed using the modified Ciuley method by Trần Hùng (2014) [8]. A 20 g sample was sequentially extracted with ethanol to assess the presence of different compound groups through characteristic chemical reactions.

- Optimization of the extraction process:

The extraction process was optimized using the Box-Behnken design in Design Expert 13.0. Independent variables included extraction temperature (60ºC, 65ºC, 70ºC), material-to-solvent ratio (1/30, 1/35, 1/40), and ethanol concentration (60%, 70%, 80%), while total polyphenol content and extraction yield were dependent variables. A total of 17 experimental runs were conducted, and the results were analyzed to identify optimal extraction conditions.

For extraction, approximately 10 g of *E. asperula powder* was accurately weighed and subjected to hot maceration for 6 hours under fixed temperatures of 60, 65, and 70°C. Ethanol was used as the extraction solvent at concentrations of 60%, 70%, and 80%, with solvent volumes adjusted according to herb-to-solvent ratios of 1:30, 1:35, and 1:40, respectively, as defined in the optimization model. Following extraction, the ethanol extract underwent chlorophyll removal by dilution and cooling at 5ºC, in accordance with the method described by Vu D. Nguyen *et al*. [9]. The resulting precipitate was filtered, and the solvent was evaporated to obtain a concentrated extract with a moisture content of less than 20%, in compliance with the Vietnamese Pharmacopoeia V standards [10].

- Quantification of total polyphenol content:

Total polyphenol content was determined using the Folin-Ciocalteu assay with UV-Vis spectrophotometry, employing gallic acid as the standard, as described by Thao N.N.N *et al*. (2022) [11]. A 0.1 g extract sample (moisture content <20%) was ultrasonically extracted at 40ºC for 30 minutes, filtered, and diluted in a 50 mL volumetric flask. A 1.0 mL aliquot was mixed with 5.0 mL Folin-Ciocalteu reagent, vortexed for 2 minutes, and reacted for 10 minutes before adding 4.0 mL of 7.5% Na_2CO_3. The mixture was incubated in the dark at room temperature for 60 minutes, and absorbance was measured at 765 nm.

A calibration curve was constructed using gallic acid solutions at different concentrations, yielding the regression equation $y=0.0094x-0.0182$ (R^2=0.9967). The experiment was conducted in triplicate to ensure accuracy.

Calculation formula: $P=\frac{a \times V}{m}$ x N x H. In which: P: Total polyphenol content (mg GAE/g dry extract); a: x-value from the gallic acid calibration curve (µg/mL); V: Volume

of the extract solution (mL); m: Mass of the extract in the solution (g); N: Moisture content of the extract; H: Extraction yield (%).

III. RESULTS

3.1. Chemical composition

Preliminary phytochemical analysis of *Ehretia asperula* ethanol extract from Can Tho revealed the presence of alkaloids, saponins, coumarins, organic acids, and polyphenols, with polyphenols being the dominant bioactive compounds. These compounds are known for their antioxidant, anti-inflammatory, and lipid-lowering effects, as well as their potential role in preventing chronic diseases, including cancer and cardiovascular disorders [12].

3.2. Optimization of polyphenol extraction

Regression analysis employs mathematical and statistical techniques to optimize experimental parameters using Design Expert 13.0 software. Table 1 presents the results of 17 experimental runs based on the Box-Behnken model, which were conducted to evaluate polyphenol content as the key response variable.

Table 1. Optimal extraction of polyphenols from *E. asperula* leaves

No.	Independent variable			Polyphenol content (mg GAE/g extract)		Extraction yield (%)	
	A: Material/Solvent (w/v)	B: Ethanol (%)	C: Temperature (ºC)	Actual yield	Predicted yield	Actual yield	Predicted yield
1	1/40	80	65	52.95	55.35	11.02	10.73
2	1/30	70	60	66.02	65.79	8.11	7.89
3	1/35	60	60	56.02	58.66	11	10.33
4	1/30	80	65	32.99	37.06	7.69	7.48
5	1/35	60	70	55.33	59.16	10.52	11.15
6	1/30	60	65	40.42	38.02	8.37	9.12
7	1/40	60	65	106.24	102.17	13.01	12.37
8	1/35	80	70	45.87	43.23	9.21	9.52
9	1/35	70	65	100.44	98.15	10.13	9.92
10	1/40	70	60	110.75	112.18	10.54	11.14
11	1/35	70	65	95.34	98.15	10.01	9.92
12	1/30	70	70	80.85	79.42	9.51	8.71
13	1/40	70	70	115.25	115.48	12.13	11.96
14	1/35	70	65	98.92	98.15	10.18	9.92
15	1/35	80	60	30.63	26.8	8.43	8.69
16	1/35	70	65	90.76	98.15	9.81	9.92
17	1/35	70	65	105.29	98.15	9.04	9.92

Based on the experimental values presented in Table 1, the lowest and highest polyphenol contents were observed in experiments 15 and 13, with values of 30.63 mg GAE/g and 115.25 mg GAE/g, respectively.

The study applied regression analysis to the experimental data and obtained a second-order polynomial model for predicting the total polyphenol content as follows: $Y_{Polyphenol}$=98.15+20.61xA-11.95xB+4.23xC-11.46xAB-2.58xAC+3.98xBC+3.14xA²-43.13xB²-8.06xC². Similarly, the extraction yield was predicted using the following model: Y_{Yield}= 9.92+1.63xA-0.8187xB+0.4113xC.

The ANOVA analysis results in Table 2, 3 indicated that the material/solvent ratio and ethanol concentration exhibited significant interaction effects ($p<0.05$) and influenced both polyphenol content and extraction yield. In contrast, temperature did not have a statistically significant effect ($p>0.05$). The lack of fit for the predictive model (polyphenol = 0.4693, extraction yield = 1.65) was not statistically significant ($p>0.05$), and the model's correlation coefficients ($R^2_{polyphenol} = 0.9847$; $R^2_{extraction\ yield} = 0.8720$) were both greater than 0,8, indicating strong predictive accuracy.

Table 2. Coefficients of variables influencing extract total polyphenol content

Source	Sum of Squares	df	Mean Square	F-value	p
Model	13582.84	9	1509.2	49.98	<0.0001
A-Material/Solvent ratio	3399.39	1	3399.39	112.58	<0.0001
B-Ethanol concentration	1141.7	1	1141.70	37.81	0.0005
C-Temperature	143.48	1	143.48	4.75	0.0657
AB	525.78	1	525.78	17.41	0.0042
AC	26.68	1	26.68	0.8836	0.3785
BC	63.44	1	63.44	2.1	0.1905
A^2	41.18	1	41.18	1.36	0.2811
B^2	7831.48	1	7831.48	259.36	<0.0001
C^2	273.53	1	273.53	906	0.0197
Residual	211.37	7	30.2		
Lack of Fit	92.04	3	30.68	1.03	0.4693
Pure Error	119.32	4	29.83		
Cor Total	13794.21	16	R^2=0.9847	CV=7.27%	

Table 3. Coefficients of variables influencing extraction yield

Source	Sum of Squares	df	Mean Square	F-value	p
Model	27.91	3	9.3	29.52	<0.0001
A-Material/Solvent ratio	21.19	1	21.19	67.24	<0.0001
B-Ethanol concentration	5.36	1	5.36	17.02	0.0012
C-Temperature	1.35	1	1.35	4.29	0.0587
Residual	4.1	13	0.3151		
Lack of Fit	3.23	9	0.3586	1.65	0.3316
Pure Error	0.8693	4	0.2173		
Cor Total	32	16	R^2=0.8720	CV=5.66%	

Note: Three-dimensional response surface plots were generated by fixing one factor at its central level (0) while varying the other two within the investigated range. These 3D plots illustrate the interaction effects of factors on polyphenol content, as depicted in Figure 1.

Based on the analysis results, the best extraction conditions for obtaining polyphenols from the concentrated extract of *E. asperula* leaves are presented in Figure 2. Under optimal conditions, 67.28% ethanol concentration, 68.91°C extraction temperature, and a material-to-solvent ratio of 1/35,157 (w/v), the polyphenol content reached 96.442 mg GAE/g extract, with an extraction yield of 10.519%.

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In this study, polyphenol extraction was conducted under experimental conditions of 69°C temperature, 67% ethanol concentration, and a material-to-solvent ratio of 1/35 (w/v). The validation results demonstrated an actual polyphenol content of 89.593 mg GAE/g extract, which falls within the confidence interval (96.442±5%) of the predicted polyphenol content from the optimized model. Therefore, the optimization model designed in this experiment is considered valid.

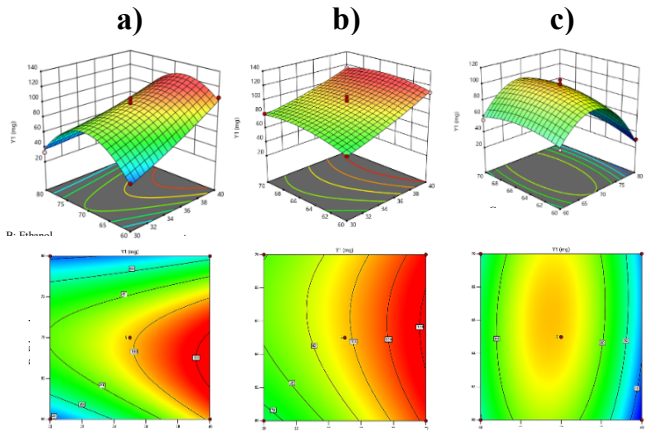

Figure 1. Response surface to polyphenol content of optimized extract from *E. asperula.*

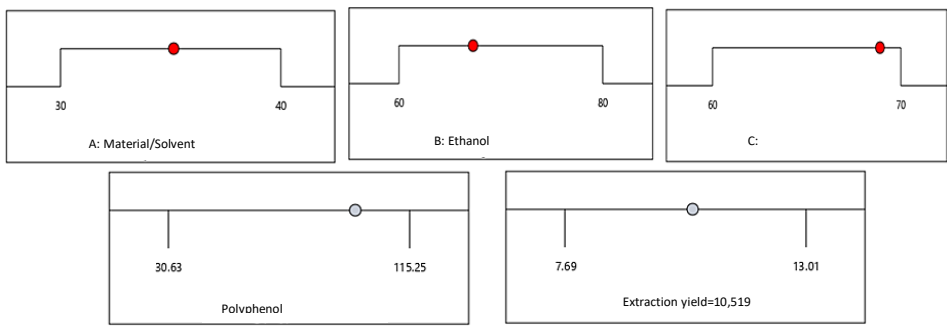

Figure 2. Expected function & optimal conditions for polyphenol content & extraction yield.

Investigation of the number of extractions

Table 4. Results of extraction cycle investigation based on polyphenol content.

Number of extractions	Polyphenol content (mg GAE/g extract)	Difference compared to extracts with fewer extractions (%)
Optimization	96.312±6.60	
2nd	46.427±9.02	48.712±12.58
3rd	10.982±1.99	7.734±1.71
4th	3.917±0.37	2.552±0.30
5th	0.398±0.20	0.254±0.13

The polyphenol content obtained in the fourth and fifth extraction cycles differed from the cumulative content of the previous extractions by 2.552% ± 0.30 and 0.254% ± 0.13, respectively, accounting for less than 2% of the total yield (Table 4). This minimal increase suggests that the majority of polyphenols were extracted within the first four cycles,

with negligible additional recovery beyond this point. Therefore, four extraction cycles were determined to be optimal for maximizing polyphenol yield while minimizing solvent usage and processing time.

IV. DISCUSSIONS

This study optimized the extraction of polyphenols from *Ehretia asperula* leaves using heated maceration with ethanol, a method that balances efficiency with simplicity while minimizing solvent consumption and extraction time. However, limitations such as solvent cost and flammability risks must be considered when scaling up. Polyphenol content was quantified using the Folin-Ciocalteu assay, a widely accepted method due to its accuracy and broad applicability compared to techniques that target specific phenolic structures [12], [13].

Optimization using the Box-Behnken design confirmed the reliability of the extraction models, with Lack of Fit values above 0.05 and correlation coefficients ($R^2 > 0.8$), aligning with findings from Tomaz *et al*. (2016) and Cui *et al*. (2019) [14], [15]. Among the extraction parameters, the material-to-solvent ratio significantly affected polyphenol yield ($p<0.05$). Increasing the ratio improved diffusion but exceeding 1/30 led to viscosity-related limitations. Ethanol concentration ($p<0.05$) showed a nonlinear effect, with optimal extraction at 70% ethanol; higher concentrations negatively impacted extraction due to protein denaturation. Temperature, within the studied range, had no statistically significant effect ($p>0.05$).

Significant interactions were observed between material-to-solvent ratio and ethanol concentration ($p<0.0001$), emphasizing the importance of optimizing these factors simultaneously. In contrast, interactions between material-to-solvent ratio and temperature, as well as ethanol concentration and temperature, were not significant. Quadratic effects, particularly ethanol concentration (B^2, $p<0.0001$), strongly influenced polyphenol yield, reinforcing the need for precise optimization. Under optimal conditions, the predicted polyphenol content was 96.442 mg GAE/g extract, with experimental validation confirming consistency within the 5% confidence interval.

Additionally, an evaluation of extraction cycles showed that after the fourth cycle, additional polyphenol recovery was minimal (2.552% ± 0.3), indicating solvent saturation. Increasing extraction cycles beyond this point was inefficient, leading to excessive solvent consumption. These findings highlight the importance of optimizing not only extraction parameters but also process sustainability by reducing solvent use and environmental impact.

V. CONCLUSIONS

The study determined the optimal extraction conditions for polyphenols from *Ehretia asperula* leaves using the Box-Behnken model (Design Expert 13.0), with an ethanol concentration of 67.28%, a temperature of 68.91°C, and a material-to-solvent ratio of 1/35.157 (w/v). The polyphenol content reached 96.442 mg GAE/g extract, with an extraction yield of 10.519%. The experimental value (89.593 mg GAE/g) fell within the confidence interval of the model. These findings provide a foundation for further investigations into the pharmacological effects of this medicinal plant in *in vitro* and *in vivo* studies, as well as for the development of polyphenol-rich formulations.

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