Study on the development of an extraction process and evaluation of certain properties of the extract from perilla (*Perilla frutescens* var. *crispa)* leaves

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ABSTRACT

Perilla (Perilla frutescens var. crispa) leaves are widely used in traditional medicine and nutrition, and are known for their richness in polyphenols and bioactive compounds. This study aimed to optimize the extraction and enrichment of polyphenols from perilla leaves collected in Dong Anh commune, Hanoi city. The optimal extraction conditions were determined as 70° ethanol, a solvent-to-material ratio of 10:1 (v/w), at 35 °C for 65 minutes, yielding 5.74 mg GAE/g dry material. The crude extract contained a total polyphenol content of 114.83 mg GAE/g, which increased to 135.16 mg GAE/g after enrichment using activated X5 resin. In addition, the enriched extract gained a rosmarinic acid content of 62.8 mg/g. These findings demonstrate that the optimized extraction process combined with X5 resin enrichment produces a polyphenol-rich extract from perilla leaves, with promising applications in pharmaceuticals and functional foods.

Keywords: Perilla leaves; Polyphenols; Rosmarinic acid; Herbal extract.

1. INTRODUCTION

Perilla frutescens var. *crispa*, a species of the Lamiaceae family, is an herbaceous plant widely distributed in Asian countries such as China, Japan, Korea, and Vietnam, where it is used as a culinary herb and in many traditional remedies. In folk medicine, its leaves are recognized for their properties in treating colds, inflammation, and pain, as well as aiding digestion [1]. Recent scientific studies have demonstrated that perilla leaves are a rich source of highly bioactive polyphenolic compounds [2]. Among these, rosmarinic acid (RA), an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid, was found in significant quantities, ranging from 3.8–5.2 mg/g of dry weight, depending on the cultivar, cultivation conditions, and harvest time [3]. The molecular structure of RA contains multiple hydroxyl groups, which contribute to its potent antioxidant capacity by neutralizing free radicals (ROS), protecting cell membranes, and inhibiting the oxidation of biological macromolecules [4]. In addition to its antioxidant activity, RA has also been shown to possess anti-inflammatory, anti-allergic, anti-diabetic, neuroprotective, anti-cancer, and immunomodulatory properties [5]. The mechanism of action of RA involves the inhibition of pro-inflammatory enzymes such as cyclooxygenase (COX) and lipoxygenase (LOX), as well as the suppression of the expression of pro-inflammatory cytokines like TNF-α, IL-1β, and IL-6 through the regulation of intracellular signaling pathways such as NF-κB and MAPK [6]. Besides RA, perilla leaves also contain various other phenolic compounds, including caffeic acid, ferulic acid, and flavonoids like luteolin, apigenin, and scutellarein. The presence of these compounds, along with RA, creates a broad spectrum of biological activities, enhancing total antioxidant capacity, inhibiting lipid peroxidation, and protecting the body against disorders related to oxidative stress, such as diabetes, arthritis, premature aging, and cardiovascular diseases [7].

Numerous methods have been employed both globally and domestically to extract polyphenolic compounds from *Perilla frutescens* leaves. Traditional extraction techniques such as maceration and Soxhlet extraction are simple and easy to perform; however, they often suffer from drawbacks including long extraction times, high solvent consumption, and relatively low extraction efficiency

[8]. To overcome these limitations, modern extraction techniques such as ultrasonic-assisted extraction (UAE) and vacuum rotation extraction have been developed and widely applied, offering advantages in reducing extraction time, improving yield, and minimizing solvent usage [9]. Frederick Lia et al. [3] reported that the polyphenol content in the methanolic extract reached the highest value of 1705.43 mg/L CAE, whereas the study conducted by Pengfei Jiao et al. [9] demonstrated that the polyphenol content in the aqueous extract was 10.94 mg/g. Nevertheless, optimization of process parameters for each specific plant material remains essential. Therefore, in this study, ultrasonic-assisted extraction was employed in combination with process optimization using the Box–Behnken design (BBD) experimental approach to accurately determine the optimal extraction conditions (including temperature, extraction time, and solvent ratio) for rosmarinic acid (RA) and other polyphenols from *Perilla* leaves, aiming to achieve the highest extraction efficiency and to contribute to the development of potential raw materials for pharmaceutical and functional food industries [10].

2. MATERIALS AND METHODS

2.1. Chemicals and equipment

2.1.1. Chemicals

The chemicals used in this study included ethanol (96°, Duc Giang, Vietnam), sodium carbonate (99.8%, Xilong Scientific, China), Folin–Ciocalteu reagent (99%, Xilong Scientific, China), rosmarinic acid (99.9%, ChemFaces, China), gallic acid (99%, Xilong Scientific, China), formic acid (99.99%, for HPLC, Merck, Germany), D101 macroporous adsorption resin (500–550 m^2/g; Miaoyang, China), and X5 macroporous adsorption resin (500–600 m^2/g; Samsung, Korea).

2.1.2. Equipment

The equipment employed in this study included a rotary evaporator (IKA RV3, Germany), a high-performance liquid chromatography system (HPLC, Shimadzu, Japan), an analytical balance (Labex, 300 g capacity, ± 0.001 g accuracy, China), filter paper (NewStar, China), a PG500 grinder (China), a TitanSonic-410 ultrasonic device (Korea), and other standard laboratory instruments.

2.2. Methods

2.2.1. Dry matter determination

The dry matter content of the perilla leaves was determined using the method of Truong Quoc Tat et al. at Tien Giang University [11] as follows: The plant material used in this study consisted of fresh perilla leaves (*Perilla frutescens* var. *crispa*) harvested in August during their fruiting stage from Dong Anh commune, Hanoi, Vietnam. The leaves were washed with clean water to remove impurities and dirt, then drained to eliminate surface moisture. Approximately 100 ± 0.05 g of fresh perilla leaves were accurately weighed and transferred to a pre-weighed stainless steel tray. The samples were dried in an oven at a temperature of 50 ± 0.2 °C. After a drying period of 4-6 hours, the samples were removed, cooled in a desiccator for approximately 15 minutes, and subsequently weighed. The drying-cooling-weighing cycle was repeated until a constant mass was achieved or the change in mass was less than 0.1%. The dry matter content was calculated as the percentage ratio of the sample mass after drying (B) to the initial fresh sample mass (A), using the formula: % Dry Matter = (B/A) × 100. The moisture content in the sample was indirectly determined by subtracting the dry matter percentage from 100. The experiment was performed in quintuplicate, and the average result was reported.

2.2.2. Experimental design for the investigation of extraction conditions

2.2.2.1. Extraction procedure for perilla leaf extract

The procedure for preparing the crude extract was performed according to the method of Tieu Thi Hong Anh et al. [12], as follows: After being cleaned and dried, the perilla leaves were ground

into a fine powder using a PG500 grinder. Exactly 20 g of the powdered perilla leaf was weighed and placed into a pre-weighed 250 mL Erlenmeyer flask. A precise volume of ethanol solvent was added, and the mixture was shaken thoroughly. The flask was then sealed and left to stand at room temperature for 24 hours. Following the maceration period, the sample was subjected to sonication at a frequency of 40 kHz for 1.0 hour. The resulting mixture was then filtered through filter paper to collect the filtrate. This filtrate was transferred to a 250 mL round-bottom flask of a known mass. The solvent was subsequently removed via vacuum rotary evaporation to yield a concentrated perilla leaf extract. The mass of the crude extract was determined by reweighing the flask after evaporation.

2.2.2.2. Design of experiments

The Box-Behnken design, a type of three-level design within Response Surface Methodology (RSM), was used to evaluate the effects and interactions of several factors on the polyphenol extraction yield from perilla leaves. Design-Expert® software was utilized to construct the experimental matrix, randomize the runs, and estimate a quadratic regression model to determine the optimal conditions. The three factors investigated were: the solvent-to-material ratio, extraction temperature, and sonication time. The experimental ranges and levels are presented in *Table 1.*

Table 1. *Box–Behnken experimental design.*

Investigated factors	Level (-1)	Level (0)	Level (+1)
A (Solvent/material ratio)	3/1	9/1	15/1
B (Temperature) (°C)	25	32	40
C (Ultrasound time) (min)	30	60	90

2.2.3. Determination of polyphenol content in the extract

The procedure for constructing the calibration curve and determining the polyphenol content was conducted in accordance with TCVN 9745-1:2013 (ISO 14502-1:2005): Determination of substances characteristic of green and black tea, Part 1: Content of total polyphenols in tea - Colorimetric method using Folin-Ciocalteu reagent [13].

2.2.3.1. Construction of the calibration curve:

Standard gallic acid solutions (20 - 200 µg/mL) were prepared in an ethanol:water (1:1, v/v) solvent. A micropipette was used to accurately transfer 1.0 mL of a standard solution and 2.5 mL of 10% Folin-Ciocalteu reagent into a test tube, which was then vortexed and kept in the dark for 5 minutes. Subsequently, 2.0 mL of a 2% (w/v) Na_2CO_3 solution was accurately added to the test tube, vortexed, and left to stand for 45 minutes at room temperature. The optical absorbance was measured at a wavelength of 765 nm, with each measurement repeated three times per sample. The calibration curve was established based on the relationship between the absorbance (OD) and the gallic acid concentration.

2.2.3.2. Determination of polyphenol content in the crude extract:

The concentrated crude extract was removed from the flask and diluted with methanol at an extract-to-methanol ratio of 1/999 (w/v). Then, 1.0 mL of the sample was accurately pipetted into a test tube, to which 2.5 mL of 10% Folin-Ciocalteu reagent was added, and the reaction was allowed to proceed in the dark for 5 minutes. Next, 2 mL of a 2% (w/v) Na_2CO_3 solution was added and vortexed. The mixture was left to stand for 45 minutes at room temperature, protected from direct light. After the reaction was complete, the OD of the reaction solution was measured at a wavelength of 765 nm using a UV-Vis spectrophotometer, with a blank sample (without the diluted extract) used for calibration. Each sample was analyzed in triplicate to ensure accuracy and reproducibility. The total phenol content in the sample was calculated from the constructed calibration curve equation.

2.2.4. Polyphenol enrichment

The polyphenol enrichment procedure was based on the process described by Zhihong Wang et al. [14] as follows: First, the resin was activated by soaking in 95% ethanol for 24 hours, followed by rinsing with distilled water. It was then sequentially treated with 1 M NaOH and 1 M HCl solutions (4 hours for each step), and finally washed again with water until neutral. For the static adsorption study, the extract was mixed with the resin at 25 °C for 2-3 hours to determine the adsorption capacity; the results indicated that 30% ethanol was the most suitable loading solvent. A diluted extract solution was prepared by dissolving 25 g of the concentrated crude extract in 700 mL of 30% ethanol. The dynamic adsorption process was performed by packing 15 g of the treated resin into a glass column. The extract was loaded at a flow rate of 2 BV/hour for 2 hours, after which the column was washed with 1.5 BV of water to remove hydrophilic impurities. Subsequently, desorption was carried out using 70% ethanol at a rate of 2 BV/hour, with a total volume of 8-10 BV used. The experiment was similarly conducted with non-activated adsorption resin to compare the efficiency of the activated resin beads.

2.2.5. Determination of rosmarinic acid content

The concentrated crude extract was removed from the flask and diluted with methanol. The rosmarinic acid content was determined according to the Perillae Folium monograph of the Pharmacopoeia Commission [15]. The method used was high-performance liquid chromatography (HPLC) with a diode-array detector (DAD) at a wavelength of 330 nm. The analysis was performed on a D18 column (2.1 × 100 mm, 1.7 µm particle size) at a column temperature of 35 °C. The mobile phase consisted of 0.2% phosphoric acid and acetonitrile under a gradient elution mode, with a flow rate of 0.5 mL/min.

3. RESULTS AND DISCUSSION

3.1. Dry matter determination

The mass of the perilla leaves after drying to a constant weight is presented in table 2.

Table 2. *Mass of perilla leaves after drying.*

Replicate	1	2	3	4	5	Average	SD	CV%
Mass (g)	13.91	14.62	12.45	14.98	13.36	13.86	0.88	6.32

The analysis showed that the average dry matter (DM) content of the perilla leaves was 13.86 ± 0.88% (n = 5). The coefficient of variation (CV) of 6.32% indicates an acceptable level of dispersion for plant materials, confirming the data's reliability. This average content means the fresh leaves contained approximately 86.14% water and ~14% dry matter. The obtained dry matter value was lower than the values reported by Natsuko Kagawa et al. [16] (26 ± 1.7%) and Rouphael et al. [17] (16.1 - 21.7% for red aromatic leaves). This difference may be attributed to factors such as the specific characteristics of the cultivation locality, including climate, humidity, and light, as well as the impact of salinity – a factor that Rouphael proved to increase dry matter accumulation. The low dry matter content observed could be explained by the local climate conditions. The hot, humid, rainy climate and non-saline soil in Northern Vietnam create optimal conditions for water absorption and foliage growth, thereby reducing the rate of dry matter accumulation.

3.2. Development of an optimized extraction process

3.2.1. Construction of the polyphenol content calibration curve using standard gallic acid solutions

The procedure for constructing the calibration curve according to TCVN 9745-1:2013 (ISO 14502-1:2005) was reported in Figure 1. The results showed that the calibration curve had a correlation coefficient of R^2 = 0.9984, demonstrating a linear relationship. This calibration equation could be used to calculate the total polyphenol content in the crude extract samples from perilla leaves.

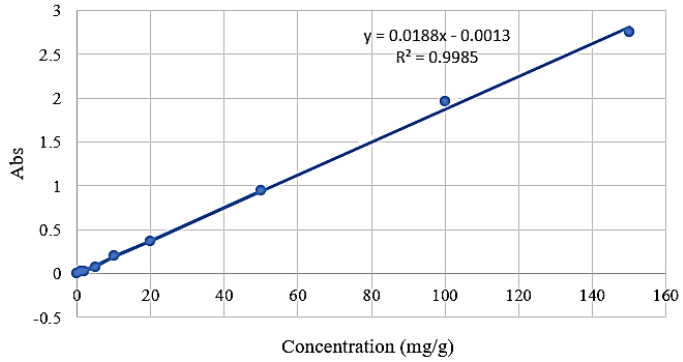

Figure 1. *Calibration curve for the determination of polyphenol content.*

3.2.2. Determination of polyphenol content in the crude extract

Table 3. *Box-Behnken experimental results for the three factors in polyphenol extraction from perilla leaves.*

No.	A	B	C	Extract mass (g)	Optical density	Polyphenol content (mg/g)	Polyphenol mass (mg)
1	15/1	32	30	1.47	1.18	63.62	93.52
2	9/1	32	60	1.31	1.723	92.50	121.18
3	15/1	32	90	2.88	0.78	42.34	121.94
4	3/1	32	30	1	1.253	67.50	67.50
5	3/1	40	60	1.31	1.042	56.28	73.72
6	9/1	25	90	1.6	1.276	68.72	109.96
7	9/1	32	60	1.33	1.741	93.46	124.30
8	3/1	25	60	1.23	1.215	65.48	80.54
9	9/1	32	60	1.17	1.992	106.81	124.97
10	15/1	40	60	2.22	0.86	46.60	103.44
11	9/1	25	30	0.97	1.331	71.65	69.50
12	9/1	40	90	1.92	1.221	65.80	126.33
13	3/1	32	90	1.08	1.333	71.76	77.50
14	9/1	32	60	1.87	1.212	65.32	122.15
15	9/1	32	60	1.43	1.607	86.33	123.45
16	9/1	40	30	1.43	1.363	73.35	104.89
17	15/1	25	60	1.47	1.253	67.50	99.23

The solvent-to-material ratio, extraction temperature, and sonication time were considered the independent variables of the process. Their individual and interactive effects on the polyphenol mass (mg) (considered the dependent variable) were investigated using the Box-Behnken experimental design. A quadratic equation to predict the optimal point was established based on the Box-Behnken design and the input variables. The empirical relationship between the dependent variable and the independent variables in coded form, based on the experimental results, is expressed as follows:

$$Y = 123.67 + 14.94\times A + 6.14\times B + 12.39\times C + 2.57\times A\times B + 4.61\times A\times C - 4.55\times B\times C - 23.27\times A^2 - 11.17\times B^2 - 9.83\times C^2$$

The ANOVA results indicated that the model's p-value was 0.0027 (< 0.05), showing the model was highly statistically significant and confirming a valid relationship between the input variables and the response. The coefficient of determination R^2 was 0.9307 and the adjusted R^2 was 0.8415,

indicating that the model explained 93.07% of the variability in the data and showed a high correlation between the predicted and experimental values. The coefficient of variation (C.V.%) of 8.40% (< 10%) demonstrated the high precision and repeatability of the experiments. However, the "Lack of Fit" p-value was 0.0006 (< 0.05), indicating that the lack of fit was significant. This suggests that while the model captures the main trends, the quadratic relationship may not perfectly describe all data points, and other factors or a different model might offer further refinement.

Table 4. *Results of the multivariate ANOVA.*

Souce	Sum of Squares	df	Mean square	F-Value	p-Value
Model	6982.61	9	775.85	10.44	0.0027
A-solvent ratio	1782.72	1	1782.72	23.99	0.0018
B-temperature	301.97	1	301.97	4.06	0.0837
C-ultrasound time	1225.06	1	1225.06	16.48	0.0048
AB	26.44	1	26.44	0.3557	0.5697
AC	84.82	1	84.82	1.14	0.3208
BC	82.97	1	82.97	1.12	0.3258
A^2	2279.24	1	2279.24	30.67	0.0009
B^2	519.51	1	519.51	6.99	0.0332
C^2	406.76	1	406.76	5.47	0.0519
Residual	520.24	7	74.32		
Lack of Fit	510.65	3	170.22	71.01	0.0006
Pure Error	9.59	4	2.40		
Cor Total	7502.85	16			

From the ANOVA results, the solvent ratio A (solvent ratio, +14.94) and C (sonication time, +12.39) indicated they have the strongest positive linear effect on enhancing the extraction yield. The coefficient for B (temperature, +6.14) was also positive but showed a comparatively smaller effect. Meanwhile, the interaction terms (AB, AC, and BC) had relatively small coefficients, suggesting they were not primary drivers of the response.

Regarding the quadratic terms, the coefficients for A^2 (-23.27), B^2 (-11.17), and C^2 (-9.83) were all strongly negative. This was significant as it showed that the relationship between the factors and the response was not linear but a parabolic curve. This also indicated that after reaching an optimal threshold, a continued increase in the value of any factor would lead to a decrease in extraction efficiency.

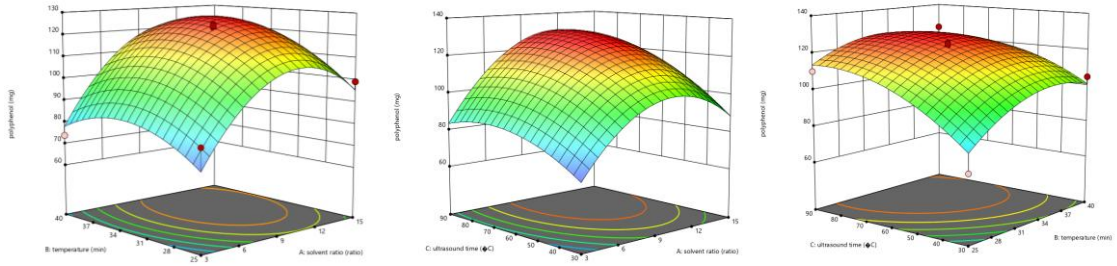

Figure 2. *Response surface plots of interactions: a) Temperature - Solvent ratio; b) Sonication time - Solvent ratio; c) Sonication time - Temperature.*

The 3D response surface plots helped to visualize the effects of the factors and identify the region for the highest polyphenol content (Y) (the red area). Based on these trends, the optimal conditions to obtain the highest polyphenol content were predicted to be: A (Solvent/material ratio): 10:1 (v/w), B (Temperature): 35 °C, and C (Sonication time): 65 minutes. The optimal

conditions were validated to ensure the accuracy of the method, and the results showed that the polyphenol content in the extract was 114.83 mg/g, equivalent to 5.74 mg/g of dry matter.

3.3. Results of polyphenol content determination after enrichment

The polyphenol content after enrichment is presented in table 5.

Table 5. *Polyphenol content after enrichment.*

No.	Sample name	Adsorbent resin	Resin mass (g)	Extract mass (g)	Optical density	Polyphenol content (mg/g)	Recovery yield (%)
1	X5-1-HP	Activated X5	15	0.54	1.319	71.01	25.20
2	X5-1-GH	Activated X5	15	1.03	2.525	135.16	48.07
3	X5-2-HP	Non-activated X5	15	0.48	1.393	74.95	22.40
4	X5-2-GH	Non-activated X5	15	1.05	2.206	118.19	49.00
5	D10-1-HP	Activated D10	15	0.67	1.807	96.97	31.27
6	D10-1-GH	Activated D10	15	0.87	2.235	119.73	40.60
7	D10-2-HP	Non-activated D10	15	0.57	1.561	83.88	26.60
8	D10-2-GH	Non-activated D10	15	0.72	2.106	112.87	33.60

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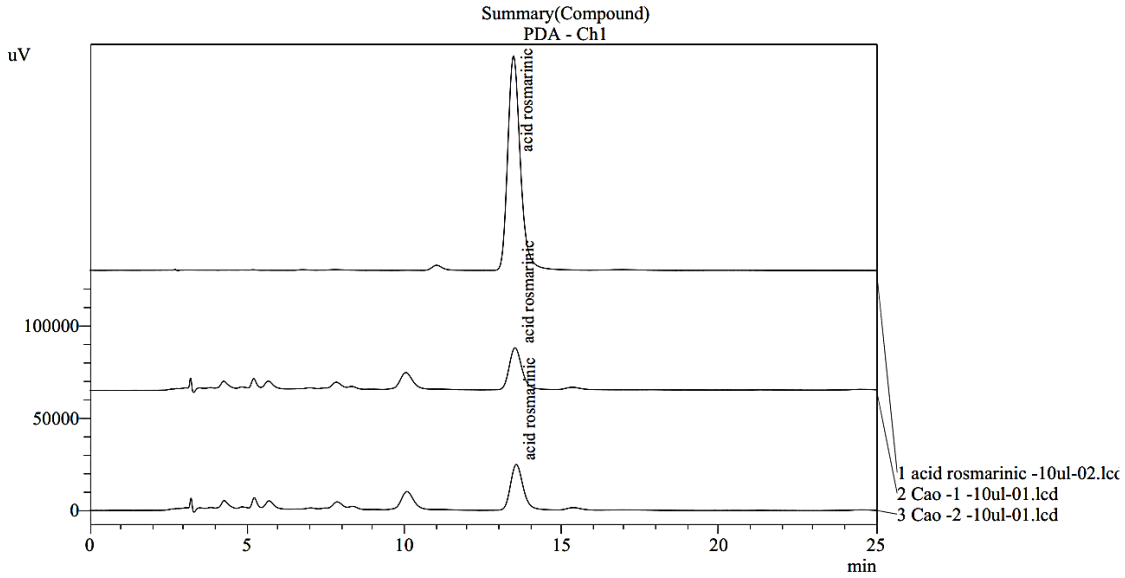

Figure 3. *HPLC chromatogram of rosmarinic acid.*

The analysis of polyphenol content showed a clear difference between the two types of resins and their activation states. With the activated X5 resin, the desorbed sample reached the highest value (135.16 mg/g), nearly double that of the adsorbed sample (71.01 mg/g), demonstrating that resin activation significantly increased the capacity to retain and release polyphenols. For the non-activated X5, the desorbed sample (118.19 mg/g) was also higher than the adsorbed (74.95 mg/g), but the difference was smaller. Similarly, the activated D10 resin yielded a higher desorption value (119.73 mg/g) than adsorption (96.97 mg/g), though the increase was not as pronounced as with X5. For the non-activated D10, the desorbed sample (112.87 mg/g) still surpassed the adsorbed (83.88 mg/g), but was lower than the activated D10. In a direct comparison, X5 consistently showed superior adsorption-desorption efficiency over D10, with higher desorption values in both states (135.16 and 118.19 mg/g compared to 119.73 and 112.87 mg/g). The X5 resin achieved

48.07% recovery in the desorption phase (the recovery yield of polyphenols reached 56.58%) and 25.20% in the adsorption phase, giving an overall yield of 73.27%, indicating efficient polyphenol recovery and practical applicability. This suggests that the structure and surface properties of X5 are more suitable for polyphenol recovery, while D10 provides stable but suboptimal results. Notably, the rosmarinic acid content after enrichment reached 62.8 mg/g, confirming the potential antioxidant properties and application value in the development of pharmaceuticals, functional foods, and natural cosmetics.

4. CONCLUSIONS

In this study, the optimal conditions for polyphenol extraction were determined to be: a solvent-to-material ratio of 10:1, a temperature of 35 °C, and an extraction time of 65 minutes, which resulted in a recovery yield of 5.74 mg GAE/g of dry material. The analysis showed that the initial crude extract contained a total polyphenol content of 114.83 mg GAE/g, and after enrichment with activated X5 adsorption resin, this value reached 135.16 mg GAE/g, demonstrating the crucial role of the enrichment process in enhancing the contents of active compounds. Furthermore, the rosmarinic acid content in the enriched extract reached 62.8 mg/g, an important indicator of the sample's quality and biological value. These data confirm that the extract is rich in polyphenol content and has potential applications in the production of pharmaceuticals and functional foods.

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TÓM TẮT

Nghiên cứu tối ưu quy trình chiết xuất và đánh giá một số tính chất của cao chiết từ lá tía tô (*Perilla frutescens var. Crispa*)

Lá tía tô (Perilla frutescens L.) là loại dược liệu quen thuộc trong y học cổ truyền và dinh dưỡng, được biết đến nhờ có chứa hàm lượng polyphenol và nhiều hợp chất có hoạt tính sinh học cao. Nghiên cứu này nhằm tối ưu hóa quy trình chiết và làm giàu polyphenol từ lá tía tô thu hái tại xã Đông Anh, thành phố Hà Nội. Quy trình chiết tối ưu sử dụng dung môi ethanol 70^o, tỷ lệ dung môi/nguyên liệu 10/1 (v/w), ở 35 °C trong 65 phút, hiệu suất thu hồi đạt 5,74 mg GAE/g nguyên liệu khô. Cao chiết ban đầu có hàm lượng polyphenol tổng số 114,83 mg GAE/g và tăng lên 135,16 mg GAE/g sau khi làm giàu bằng nhựa X5 đã hoạt hóa, đồng thời hàm lượng rosmarinic acid trong cao chiết đạt 62.8 mg/g. Kết quả nghiên cứu cho thấy, quy trình chiết tối ưu kết hợp với làm giàu bằng nhựa X5 tạo ra cao chiết giàu polyphenol từ lá tía tô, mở ra tiềm năng ứng dụng trong sản xuất dược phẩm và thực phẩm chức năng.

Từ khóa: Lá tía tô; Polyphenol; Rosmarinic acid; Cao chiết dược liệu.