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Optimization of ultrasound-assisted extraction using box-Behnken design, method validation, and analysis of phytochemicals from drying treatments in the application of *Etlingera elatior* inflorescence

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ABSTRACT

Background: *Etlingera elatior* inflorescence, a potential source for herbal tea, undergoes a drying process that could enhance the active ingredients and health benefits. This study introduces the use of ultrasound-assisted extraction (UAE) to improve conventional extraction and increase the yield of phytochemicals, thereby presenting a novel approach to functional foods and nutrition.

Objective: This study aims to optimize the UAE method using the Box-Behnken Design (BBD), a statistical experimental design widely used in the field of functional foods and nutrition. The determination of phytochemicals was validated using the optimized UAE. Chlorogenic acid from the extract was analyzed using HPLC-DAD and compared with that from Indonesia. According to the development of herbal tea, the phytochemical contents and inhibition of DPPH scavenging antioxidant activity were examined for the three drying treatments compared to freeze-drying.

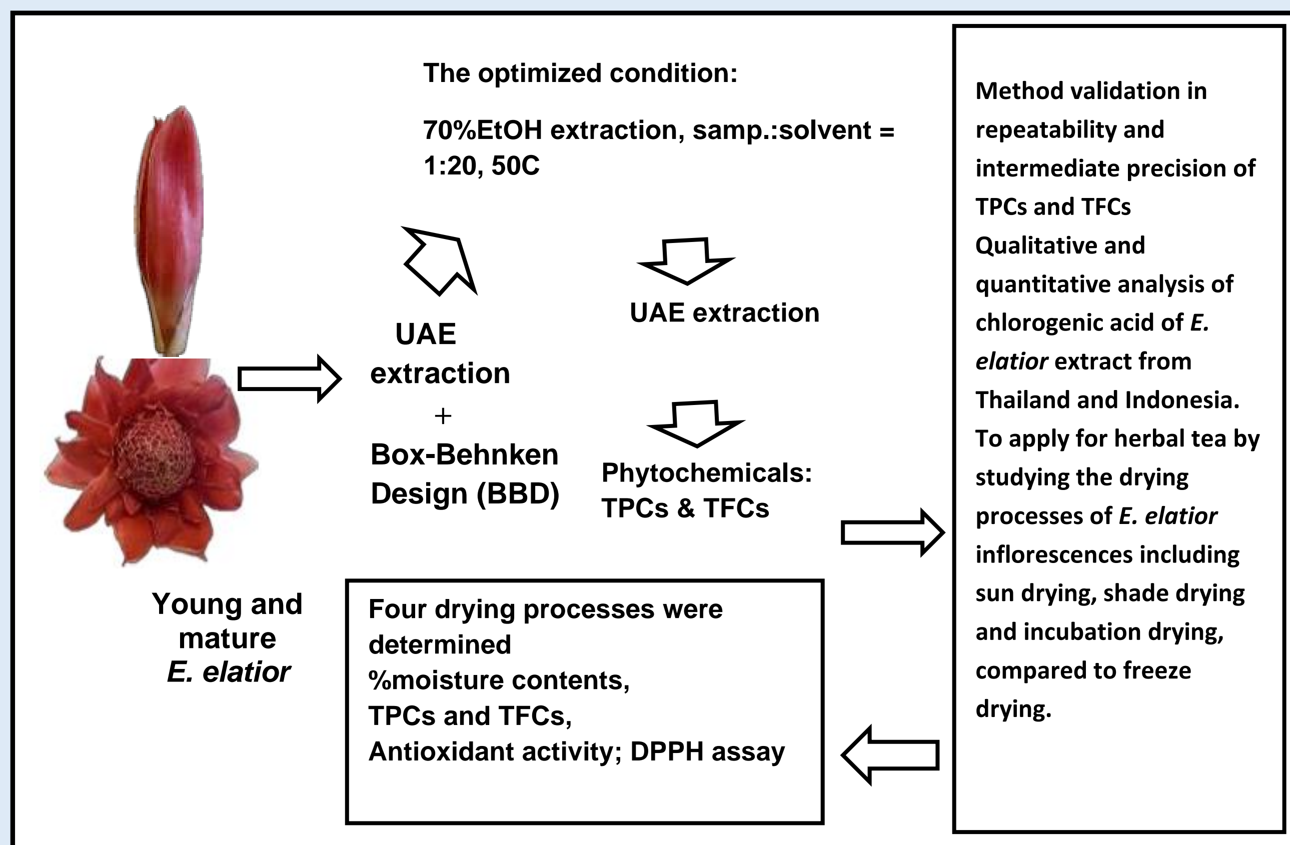
Methods: Box-Behnken Design (BBD) was used to optimize the condition of UAE with three variables: solvent concentrations (70%, 85%, 100%), solid-to-solvent ratios (1:20, 1:15, 1:10), and temperatures (30°C, 50°C, 70°C). The TPCs and TFCs of the ethanol extract were analyzed as observed responses. Repeatability precision (%CV) and recovery

were validated for the optimized UAE. HPLC-DAD analyzed the chlorogenic acid of the extract and compared it to the Thai *E. elatior* extract and Indonesia. In applying *E. elatior* tea, phytochemical contents, and DPPH·, scavenging antioxidant activity was examined for three drying treatments and compared to the freeze-drying treatment.

Results: The suggested condition of optimized UAE extraction was 70% ethanol, 50°C, and a 1:20 solid-to-solvent ratio. %Repeatability precision of TPCs and TFCs for the extract was significantly <5%, and %recovery >90%. Freeze-drying and cabinet-drying treatments had significantly higher TPCs than others. Especially in freeze-drying treatment, the TFCs of the extract from Indonesia were significantly greater than those in Thailand ($p<0.05$). For DPPH· antioxidant activity, all treatments except freeze-drying of the extracts from Indonesia had higher inhibition of DPPH· antioxidant activity than those from Thailand.

Conclusion: The optimized UAE extraction method was validated with high precision and accuracy. All drying treatments influenced the phytochemical contents and Inhibition of DPPH antioxidant activity in the inflorescences of both Thailand and Indonesia.

Keywords: Box-Behnken Design, drying treatment, tea, method validation, phenolic.



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INTRODUCTION: *Etlingera elatior (Jack) R.M. Sm.* (*E. elation*) or torch ginger, an herb belonging to the Zingiberaceae family, is widely grown in tropical regions such as Malaysia, Indonesia, Thailand, and Brunei. The most consumed part of the torch ginger plant is its inflorescence. *E. elatior* inflorescences could be used as a flavor enhancer, culinary, ornamental flowers, and traditional medicine [45]. Such inflorescence can develop into herbal tea. Drying is the first step in the production of herbal tea. The most popular techniques include hot air, shade, and sun drying because they are less expensive [32]. The different temperatures of various drying processes could affect their phytochemicals, that is, antioxidant properties and polyphenol substances, as they might influence the change in the chemical composition and antioxidant properties [48]. The active ingredients obtained from a suitable drying process would enhance tea's characteristics and human benefits [63].

E. elatior inflorescences contain phytochemicals that can be potential functional food for preventing health problems such as anti-inflammatory [19], cardiovascular disease prevention [19], obesity control, anti-carcinogenic, and diabetes alleviation properties [67]. The starting point for analyzing the phytochemicals contained in the flowers was extraction. Extracting phytochemical compounds provides two main strategies: conventional methods and techniques that assist the extraction. Traditional extraction methods include hydro-distillation, soxhlet extraction, and maceration. The disadvantages of conventional methods include higher costs and longer processing times [3].

Among the various assisted extractions (i.e., ultrasound and microwave-assisted extraction, as well as supercritical fluid extraction), ultrasound-assisted extraction (UAE) is an eco-friendly extraction method with environmental benefits, minimal chemicals, and energy use. This technique uses ultrasound waves to produce cavitation until the cell walls are broken. The bioactive compounds diffuse through the cell wall and are washed out into the solvent—the ultrasonically induced cavity results in increased diffusion through the cell membrane and mass transfer [10]. Since bioactive compounds extracted by UAE have less degradation, shorter extraction time, and higher extraction yield [33], UAE was then considered to extract the phytochemicals of interest for the objective of this study. The main factor of extraction by UAE should consider several parameters, including the polarity between the bioactive and solvent, %amplitude and frequency, extraction time, and temperature. Therefore, this study selected polar solvents such as acetone, methanol, ethanol, and water because of their polar phytochemical components (i.e., phenolic and flavonoid compounds) [55]. Moreover, the extraction temperature may have affected the results. High temperatures during the extraction process might cause more rapid hydrolysis of cell walls, increasing the release of bioactive components [54]. The hydrolysis of the cell wall is related to the solid-to-solvent ratio. Increasing the volume of solvent used for extraction leads to an increase in ultrasonic intensity, thus enhancing the mass transfer of bioactive compounds from the cell to the solvent [58]. A Box-Behnken statistical experiment Design using response surface methodology (RSM) was employed to optimize UAE extraction conditions. The parameters influencing the UAE extraction process were screened and optimized. Box-Behnken design (BBD) is an independent, rotatable quadratic design with no embedded or fractional factorial points between the variable space and the center [40]. RSM utilizes quantitative data to evaluate several parameters, statistically optimizing UAE extraction. Hence, RSM combines statistical and mathematical methods to generate mathematical models [57].

Chlorogenic acid (CGA) is a naturally occurring polyphenolic organic acid that exerts various

pharmacological effects, including antioxidant [26, 55], anti-inflammatory [23], antibacterial [28], anti-carcinogenic [6], anti-obesity, and hepatoprotective activities [34]. Furthermore, CGA regulates lipid and glucose metabolism; thus, it could lower cholesterol and triglyceride levels in the plasma of animal models induced with a high-fat diet. The beneficial effects of CGA can be used to treat hepatic steatosis [53] and type II diabetes mellitus [64]. Among the various pharmacological activities, CGA has shown relatively low toxicity and side effects [13, 61]. Chlorogenic acid is responsible for its strong antioxidant action by scavenging free radicals, inhibiting lipid oxidation, and chelating metal ions. It can scavenge free radicals at a higher rate than other phenolics, and its phenolic OH group is a metal binding site [11, 49]. In this study, in the analysis of phenolic contents in *E. elatior* inflorescences using HPLC-DAD, a high concentration of chlorogenic acid for both Thailand and Indonesia *E. elatior* appeared at λ_{325} nm. The wavelength used in this study to analyze chlorogenic acid using HPLC-DAD was 325 nm. This is based on previous studies [43, 52], which confirmed that chlorogenic acid was detected at a wavelength of 325 nm using HPLC.

For the preliminary study, the BBD-RSM factorial design was used to optimize the extraction parameters of solvent concentration (X_1), the ratio of sample and solvent (X_2), and extraction temperature (X_3) to maximize the recovery of total phenolic and flavonoid contents in dried *E. elatior* inflorescence. Using this optimized condition in the BBD domain, we validated the phytochemical content and the precision, accuracy, and recovery of the phytochemical content. In addition, phenolic compound (chlorogenic acid, mg g^{-1} sample) was identified using a High-Performance Chromatography-Diode Array Detector (HPLC-DAD) compared to the chlorogenic acid content of *E. elatior* Thailand and Indonesia. Making *E. elatior* inflorescence tea was related to the drying process of the bracts. The drying technique influences the phytochemical contents. The effects of drying techniques (i.e., sun, shade, cabinet, and freeze-drying) on young and matured inflorescences were compared between *E. elatior*, Thailand, and Indonesia. The TPC and TFC contents on young and mature inflorescences were also determined for comparison.

MATERIALS AND METHODS

Materials: Fresh *E. elatior* flowers were obtained from local farmers in Indonesia (Sleman, Yogyakarta, Indonesia) and Thailand (Sadao, Songkhla, Thailand) at harvest in June 2023. After washing and sorting with water, the fresh flowers were dried using a freeze-dryer. The dried flowers were ground and sieved through a 45-mesh sieve. Flower powder was stored in an airtight container at 4°C.

To prepare an accurate sample, E. elatior inflorescences were separated from the flower stalks. Then, the sample was cleaned with tap water and dried using three techniques (cabinet dryer (50°C, 5 days), sun drying (32-45°C, 5 days), and shade drying (30-32°C, 5 days)). The dried flowers were extracted using the UAE-optimized extraction method, performed in triplicate.

Chemicals: The chemicals and reagents used in this study included absolute ethanol, methanol, acetone, HPLC grade methanol, and NaOH (RCI Labscan Limited, Bangkok, Thailand). $NaNO_2$, $AlCl_3$, Na_2CO_3 (Ajax Finechem Pty. Ltd, New Zealand, Australia). Analytical grade standard compounds (gallic acid, quercetin hydrate, chlorogenic acid, 2,2-Diphenyl-1-picrylhydrazyl, Trolox methyl ether) (Sigma Aldrich Chemical Co., St. Louis, USA). Trifluoroacetic acid (Fisher Scientific, Loughborough, UK). Folin-Ciocalteu (Loba Chemie Pvt. Ltd., Mumbai, India).

METHODS

Ultrasound-assisted extraction (UAE): An ultrasonic system UIP1000hdT ultrasonic processor (1000 W, 20 kHz) with BS4d40 probe (Hielscher Ultrasonics GmbH, Teltow, Germany) was used to assist the extraction. Each sample was weighed (3 g) and placed in a glass beaker. Extraction was performed at 50% amplitude and 50 W power for 15 min. After extraction, the crude extracts were centrifuged (Mikro 22R, Hettich Zentrifugen, Bangkok, Thailand) at 6000 rpm for 15 min. The supernatant was filtered through Whatman filter paper No.1, kept in closed vials, and stored at 4°C until analysis.

Experimental design: In this research, a Box-Behnken Design (BBD) was used for UAE Optimization. BBD uses three levels (-1 (low), 0 (medium), 1 (high)) and three factors (X_1: solvent concentration, X_2: ratio sample to solvent, X_3: temperature) in combination with TPC and TFC. The total number of experimental units was 15, with three center points. Once the BBD was completed, Minitab software (Minitab Ltd, Brandon Curt, UK) was used for data analysis. The statistical significance of the studied factor and the evaluation of the fitting quality of the polynomial model were defined based on an analysis of variance (ANOVA). After the response surface equation from the developed models on the response over the BBD domain, multi-response optimization (MRO) was used to simultaneously optimize the two responses (TPC and TFC). Based on the RSM, a desirability function was constructed for each response to obtain the best compromised optimum condition.

Determination of phenolic compound by HPLC-DAD: The extracts were analyzed using high-performance liquid chromatography 1200 series (Agilent Technologies, Germany) with diode array detector G1315D. The chromatographic conditions were Agilent Poroshell 120 EC-C18 (4 µm, 4.6 × 150 mm). The column temperature was set to 25°C. The mobile phase consisted of phase A (0.1% trifluoroacetic acid (TFA) in water) and phase B (100% methanol) according to the following linear gradient (time, % solvent B): 0 min, 0%; 10 min, 10%; 20 min, 25%; 30 min, 60%; 40 min, 70%; and 45 min, 0%. The flow rate was 0.8 mL min^{-1}. The extracts were filtered through a 0.2 µm nylon syringe filter (Finetech Research and Innovation Corporation, Taichung, Taiwan) before injection into the chromatographic system. The volume of injection for extracts and standards was 15 µL, and the chromatograms were observed at λ_{325} nm.

Method validation: The precision and accuracy of the developed UAE were assessed. Precision was expressed as repeatability precision (%CV) and evaluated at two levels: repeatability and intermediate precision. For the repeatability analysis, nine extractions were conducted on the same day. For the intermediate precision study, three extractions were completed on each of three consecutive days (a total of nine experiments). The extraction was repeated for up to three cycles to ensure complete recovery and calculate the %recovery. In the first extraction cycle, the supernatant was collected after centrifugation, and the dried flower residue was re-extracted with fresh solvent for the second and third extractions, respectively. The TPCs and TFCs of the extracts obtained from each extraction cycle were measured. The experiments were performed in triplicate. The recovery was calculated by summing up several cycles' extraction results. The first cycle results were divided by the total extraction results multiplied by 100%. The coefficient of variation (CV; %) was calculated by dividing the standard deviation by the mean value multiplied by 100%.

Total phenolic content (TPC): The TPC of samples was determined using the Folin-Ciocalteau method [9]. Approximately 100 µL of each sample was mixed with

950 µL of Folin-Ciocalteau reagent, diluted with distilled water. After incubation at 25°C for 8 min, the mixture was combined with 950 µL of 7.5% Na_2CO_3 and incubated at 25°C for 30 min in dark conditions. The absorbance of the supernatants was measured at λ_{765} nm using a microplate reader (BioTek PowerWave XS, Winooski, VT, USA, Gen5 1.09 software), with gallic acid as a reference standard. The results were expressed as gallic acid equivalents (mg GAE g^{-1} dry matter).

Total flavonoid content (TFC): The TFC of the samples was determined using the aluminum chloride method [46]. Five hundred microliters of each sample were mixed with 150 µL of 5% $NaNO_2$. After incubation at 25°C for 6 min, the mixture was combined with 300 µL of 10% $AlCl_3$ and 500 µL of 1M NaOH. The supernatant was shaken gently and incubated for 10 min. The absorbance of the supernatant was measured at λ_{510} nm using a microplate reader (BioTek PowerWave XS, Winooski, VT, USA, Gen5 1.09 software), with quercetin as a reference standard. The results were expressed as equivalents of quercetin (mg QE g^{-1} dry matter).

Antioxidant activity: The antioxidant activity was determined using the DPPH$^{\cdot}$ scavenging activity assay [7]. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was prepared at 0.2 mmol L^{-1} in absolute methanol. Briefly, 100 µL of the sample was mixed with 100 µL of DPPH in a 96-well microtiter plate. The reaction mixtures were incubated at room temperature for 30 min before measuring absorbance at λ_{517} nm. Trolox reagent was used as the reference standard. The antioxidant activity of the samples was determined by the percentage of DPPH$^{\cdot}$ inhibition using the following formula:

%inhibition of DPPH$^{\cdot}$

$$= \frac{A\ Control - A\ Sample}{A\ Control} \times 100\%$$

$$= \frac{A\ Control - A\ Sample}{A\ Control} \times 100\%$$

Where A control is the absorbance of the DPPH solution without extract

A sample is the absorbance of the sample with DPPH solution.

The drying treatments of *E.elatior* inflorescences in the application:

Sun drying treatment: *E. elatior inflorescences were separated into bracts, placed into plastic baskets, and drained of water for one day. The bracts were dried in sunlight for five* days.

Cabinet drying treatment: The clean bracts were placed in plastic baskets**,** and water was drained for one day. They were then kept in a cabinet oven at 50 °C for five days.

Shade drying treatment: After the clean bracts were drained of water for one day**,** they were dried under a canopy with bright light for 5 days.

Moisture content: A moisture analyzer determined the moisture content [46]. Briefly, 1–2 g of *E.elatior* bract was placed into a plate of moisture analyzer, and the moisture content after evaporating the water at a temperature of 105°C.

Statistical analysis: The data were repeated in triplicate and presented as mean ± standard deviation. One-way ANOVA was implemented to analyze the variance. Fisher's Least Significance Difference (LSD) test used the analysis of variance for the difference between means in the pairwise comparison technique. +. The *p-value* less than or equal to 0.05 is considered statistically significant.

RESULTS AND DISCUSSION

In the preliminary study, screening conditions for UAE extraction involved two independent variables (solvent

types and temperatures). Subsequently, the optimum solvent type and temperature were used for the UAE extraction. The complete UAE conditions were optimized using BBD, which included three variables: solvent concentration (X_1), solid-to-solvent ratio (X_2), and temperature (X_3). The BBD was applied to the UAE condition to obtain the highest TPC and TFC, and the influence of each variable was evaluated as statistically significant using a Pareto chart.

Solvent screening: Four solvents, including water, absolute ethanol, methanol, and acetone, were considered for extracting phytochemicals to determine the optimal UAE condition. The UAE condition was operated at a 50% amplitude, 50 W power, and a solid-to-solvent ratio of 1:20, for 15 min. The results showed that the type of solvent influences the TPC and TFC. The lowest to highest order of TPC obtained from extraction with water, methanol, acetone, and absolute ethanol were 5.53±0.03, 6.94± 0.07, 11.96±0.20, 12.85±0.12 mg GAE g^{-1}. The lowest to highest order of TFC resulted from extraction with acetone, water, methanol, and absolute ethanol were 3.19±0.34, 5.96±0.02, 8.04±0.34, 10.73±0.07 mg QE g^{-1} (Figure 1). It was concluded that ethanol is the solvent type with the highest TPC and TFC among the other solvents. Several types of solvents (water, absolute ethanol, methanol, and acetone) have various levels of polarity, and the polarity of the solvent affects the solubility of the compound and the amount of yield extracted during the extraction process [20]. Phenolic and flavonoid compounds in *E. elatior* inflorescence have a polarity close to absolute ethanol. Therefore, ethanol solvent is effective for extraction. This follows previous research [55], which states that *E. elatior* inflorescence extract from ethanol had a high bioactive content. The similarity of polarity properties with the solvent used for extraction facilitates the dissolution of the bioactive material.

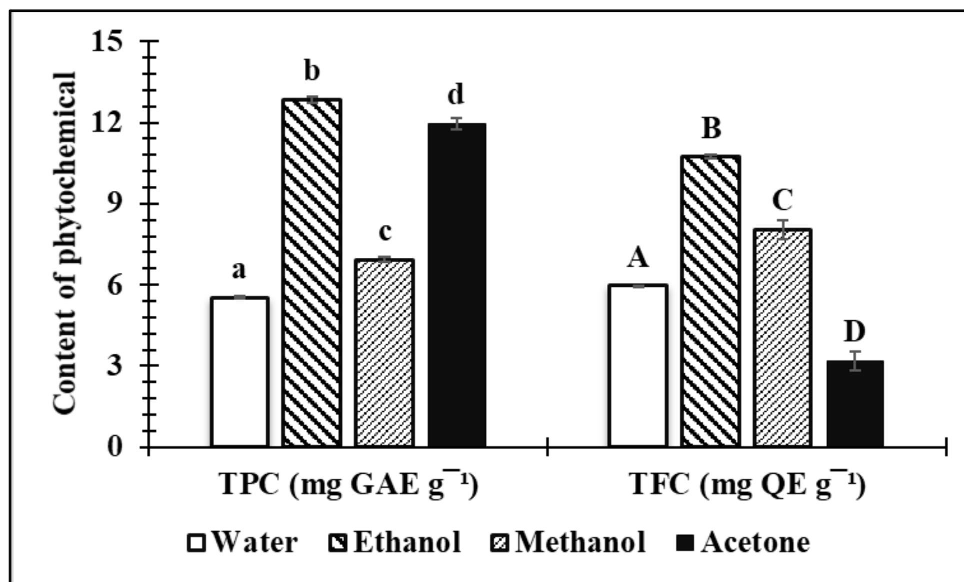


Figure 1. Total phenolic and flavonoid contents in extracts from *E. elatior* powder using various solvents: water, absolute ethanol, methanol, and acetone. The standard deviation is displayed as an error bar. A different small letter in different bar graphs means significant differences based on Fisher's Least-Significant Difference (LSD) ($p < 0.05$).

Temperature screening: Three studied temperatures (i.e., 30°C, 50°C, and 70°C) were extracted at a 50% amplitude, 50 W power, and solid-to-solvent ratio of 1:20 with a selected solvent for 15 min. The results showed that the temperature influences the TPC and TFC. The range of temperatures used for extraction was 30-70°C, which was also used in previous studies [3, 51, 65]. According to Figure 2, TPC obtained from the extraction with 30°C, 50°C, and 70°C were 10.20±0.03, 11.75±0.07, 11.16±0.19 mg QE g^{-1}. TFC obtained from extraction with 30°C, 50°C, and 70°C were 10.12±0.595, 13.77± 2.03, 13.42±0.79 mg QE g^{-1}. TPC and TFC were increased at 30-50°C, and when the temperature reached 70°C, TPC and TFC decreased. The highest result of TPC and TFC was at 50°C. This result was relevant to a previous study [21] that reported that the highest flavonoid content in dried *Dendrobium chrysotoxum* flower extraction was at 50°C because increasing temperature could lead to degradation of the phenolic and flavonoid compounds. Another study [20] reported that the temperature of 60°C was the highest limit for the extraction process because exceeding this point will decrease the stability of phenolic and flavonoid compounds.

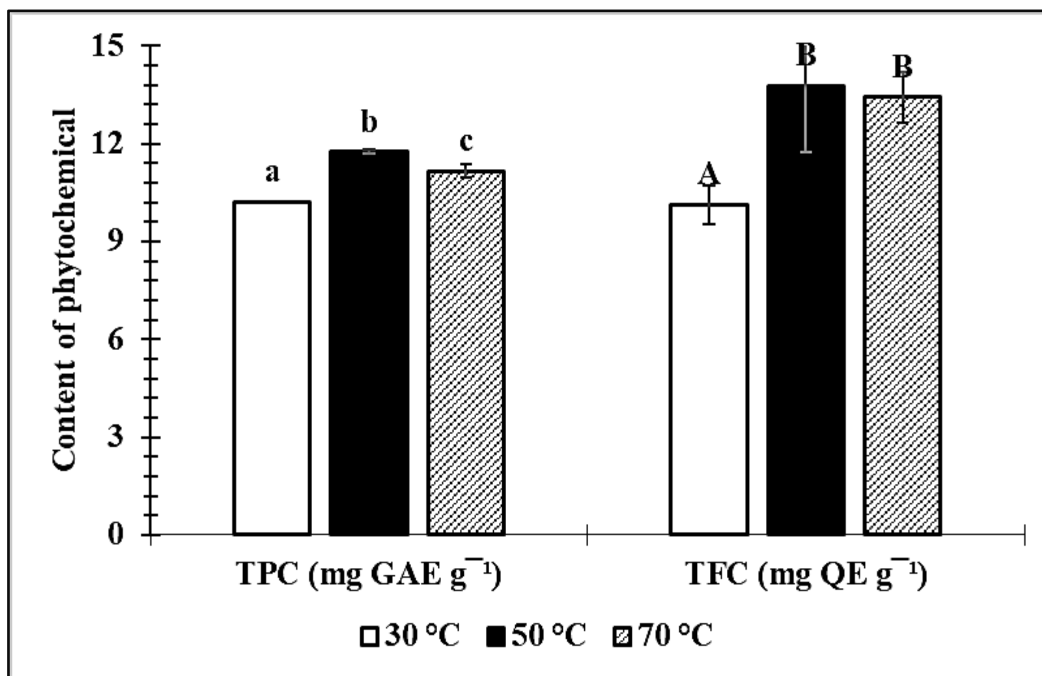


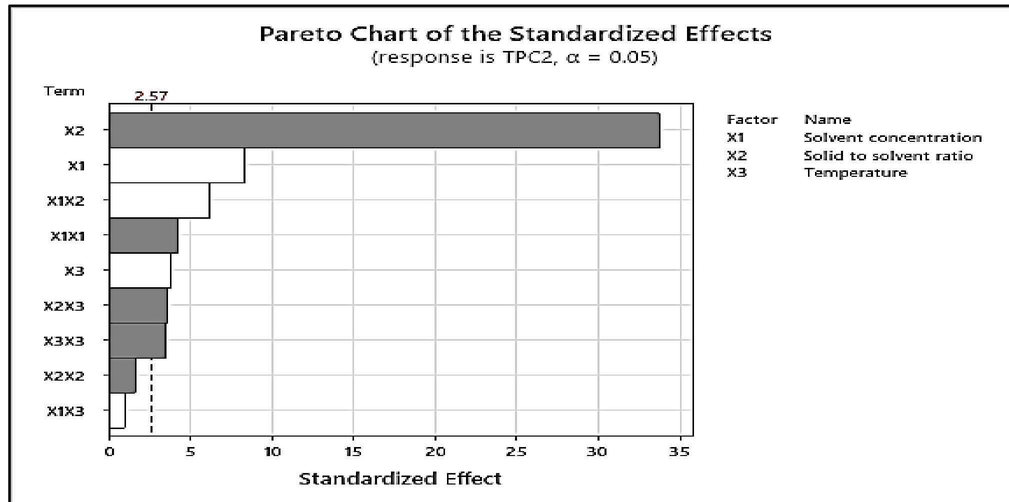
Figure 2. Total phenolic and flavonoid content in an ethanol extract from *E. elatior* powder using various temperatures: 30°C, 50°C, and 70°C. The standard deviation is displayed as an error bar. A different small letter in different bar graphs means significant differences based on Fisher's Least-Significant Difference (LSD) ($p < 0.05$).

Optimization of the UAE method: The optimization of the UAE method is based on BBD according to three variables: solvent concentration (X1), ratio sample to solvent (X2), and temperature (X3). A BBD was generated based on 15 experiments with 3 replicates, a three-factor (-1, 0, +1), and three independent variables (X_1, X_2, X_3), as shown in Table 1. Analysis of variance (ANOVA) was conducted to analyze the model's statistical significance, the impact of the variables, and any potential interactions between them. The acceptance in the error value should not exceed 10% [24]

Table 1. Box-Behnken design with normalized measured responses and prediction error.

RUN	Factor			TPC (mg GAE g^{-1})		X_3	TFC (mg QUE g^{-1})		Predicted
	X_1	X_2	-1	Observed	7.50		Observed	Predicted	
1	0	-1	0	7.54	8.73	0.56	9.48	9.26	2.39
2	-1	-1	+1	8.61	6.60	1.33	10.40	10.32	0.75
3	-1	0	0	6.67	6.64	0.99	6.84	7.01	2.43
4	0	0	+1	6.79	4.53	2.31	9.99	9.64	3.63
5	0	+1	+1	4.49	5.92	0.92	5.79	6.01	3.69
6	+1	0	0	5.85	4.57	1.26	8.75	8.45	3.54
7	+1	+1	0	4.68	6.64	2.55	7.45	7.53	1.03
8	0	0	0	6.51	7.04	1.99	9.53	9.64	1.08
9	+1	-1	-1	7.06	5.41	0.34	7.40	7.79	5.03
10	+1	0	-1	5.35	6.37	1.21	7.64	7.47	2.28
11	-1	0	0	6.44	4.52	1.17	7.41	7.71	3.88
12	-1	+1	0	4.49	6.64	0.53	4.18	3.79	10.34
13	0	0	+1	6.62	8.37	0.32	9.39	9.64	2.55
14	0	-1	-1	8.42	4.66	0.61	9.88	9.79	0.95
15	0	+1	-1	4.61	4.66	1.09	6.16	6.25	1.48

The BBD was then applied, and the *p*-values were calculated according to the t-test using Minitab software at a 95% confidence level; this means that the variables with *p*-values below 0.05 were considered influential. The influence of each variable was graphically represented in a Pareto Chart. According to a Pareto chart, the chart shows the absolute values of the standardized effects from the largest, most significant effect to the smallest effect. This chart also created a reference line indicating statistically significant effects. The reference line for statistical significance depended on the significance level. For the study, it could be observed that the most influential variables from the three parameters represented solvent concentration (X1), solid-to-solvent ratio (X2), and temperature (X3), as shown in Figure 3.



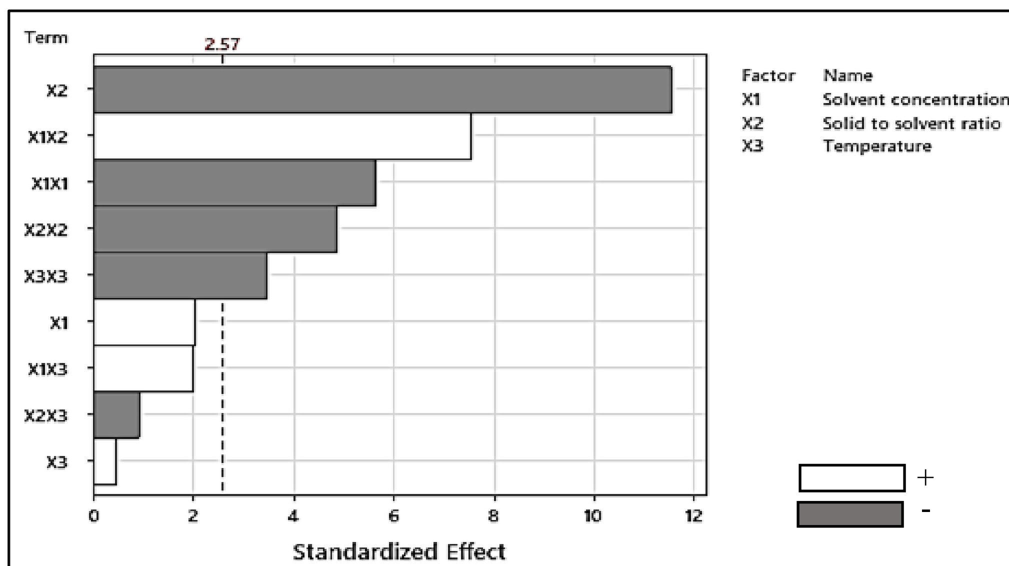


Figure 3. Pareto chart for the standardized effect of the variables on the UAE of (a) TPC, (b) TFC

The solid-to-solvent ratio had a negative effect on the two responses, which indicated that the polyphenols content decreased if a larger solvent-to-solid ratio was used. Hence, a lower sample-solvent ratio increases the contact area between the surface of the plant material and the solvent, resulting in increased phytochemical extraction. However, a previous study [22] demonstrated that using a high volume of solvent for the extraction procedure could negatively impact phytochemical extraction. Additionally, if the solvent is insufficient, it may inhibit the cavitation effect of the ultrasound extraction. The equilibrium points of the sample-solvent ratio may differ from several types of solvents and plant materials [36].

The solvent concentration had a positive effect on two responses. The polyphenol content increased with increasing solvent concentration. The polarity of the solvent is related to the extraction of the phytochemical components. Polar solvents, such as water and ethanol, are frequently used to extract polar compounds. Another study [68] suggested that the polyphenol compounds in *E. elatior* are more soluble in ethanol. In contrast, the

quadratic effect of solvent concentration negatively influenced the two responses. The high solvent concentration might have adverse effects, such as reducing the compound's solubility. Therefore, water can be added to determine the optimal concentration. The previous study reported that adding water to organic solvents such as ethanol could increase the polarity of the solvent [20]. Another study [33] reported that the polarity of solvents and specific bioactive compounds is related. When the polarity of the extraction solvent closely fits the targeted bioactive compound, the extraction process from plant cells is efficient.

Furthermore, the temperature had a positive effect on the two responses. An increase in temperature can increase the mass transfer rate and solubility owing to softening of the plant matrix and an increase in the kinetic energy of the molecules, which facilitates the diffusion of plant chemicals into the solvent. In contrast, the quadratic effect of temperature negatively influenced the two responses. The previous study [8] suggested that it is necessary to be concerned about the degradation of polyphenols at high temperatures. In addition, excessive temperatures can lead to solvent evaporation during extraction because extraction occurs at temperatures close to the solvent's boiling point [20].

Optimization of phytochemicals extraction from *E. elatior* inflorescences based on the coefficients of each variable was obtained using a second-order polynomial equation. The mathematical model can be applied to predict the levels of phytochemical compounds under any experimental conditions. A complete quadratic polynomial equation was used to verify the significance of each variable. The equations for the model obtained for TPC (1) and TFC (2) are as follows:

$$Y = 7.38 + 0.1083X_1 - 111.7X_2 + 0.0910X_3 - 0.001375X_1^2 - 189X_2^2 - 0.000637X_3^2 + 1.156X_1X_2 + 0.000229X_1X_3 - 0.499X_2X_3 \quad (1)$$

$$Y = -9.53 + 0.561X_1 - 150.3X_2 + 0.1022X_3 - 0.005438X_1^2 - 1692X_2^2 - 0.001886X_3^2 + 4.181X_1X_2 + 0.001396X_1X_3 - 0.384X_2X_3 \quad (2)$$

This finding confirmed that the optimized UAE condition using BBD and Pareto chart with statistical significance at a 95% confidence level was more effective than conventional UAE because it improved the highest TPC and TFC of extraction and use in a short time. This suggested that 71.21% ethanol in water was the extraction solvent, 48.18°C of extraction temperature, and 1:20 of solid-to-solvent ratio.

Table 2. Predicted and actual response values in the optimum extraction condition.

	Solvent concentration (%)	Solid-to-solvent ratio	Temperature (°C)	TPC (mg GAEg^{-1})	TFC (mg QUEg^{-1})
Predicted	71.21	0.05	48.18	8.67	10.41
Actual	70.00	0.05	50.00	9.14±0.11	11.09±0.21
% Error	1.70	0	3.77	5.42	4.32

In addition, by confirming the optimum condition of extraction from Minitab software at a 95% confidence level and the actual extraction, it was determined that the optimum extraction conditions were 70% ethanol in water as the extraction solvent, 50°C of extraction temperature, 1:20 of solid-to-solvent ratio. The ethanol

concentration was determined according to a previous study [27], which stated that an ethanol concentration of 70% was the optimal solvent concentration for extracting *Lithocarpus litseifoliu*, an herbal tea. The total phenolic content obtained from extraction at optimum conditions was 9.14±0.11 mg GAE g^{-1} dried sample. The total flavonoid content obtained from extraction at optimum condition is 11.09±0.21 mg QE g^{-1} dried sample (Table 2).

Analytical method validation: The optimized UAE extraction for TPCs and TFCs was validated by examining the repeatability, intermediate precision, and recovery values. Following the Guidelines for standard method performance requirements [2], it also satisfied the analyte concentration of 0.1% and acceptable recovery of 95-105%. Based on the results of the recovery with the optimum extraction condition obtained for the first cycle, the recovery value of TPC was 96.334% (9.69±0.03 mg GAE g^{-1}), and that of TFC was 94.289% (15.40±0.06 mg QE g^{-1}), as shown in Table 3. According to validation guidance [2], it had been considered that the first extraction cycle for TPC and TFC could meet satisfactory requirements.

Table 3. The contents of phytochemicals extracted from three cycles of optimized UAE on *E. elatior* inflorescence.

Phytochemical Parameters	UAE extraction content		
	Cycle 1	Cycle 2	Cycle 3
TPC (mg GAE g^{-1})	9.69±0.03	0.37±0.06	ND
TFC (mg QE g^{-1})	15.40±0.06	0.89±0.02	0.05±0.02

Precision has two different meanings: repeatability and intermediate precision. The repeatability value was obtained by measuring the sample within one day nine times per replication. For intermediate precision (reproducibility), the precision value was obtained by measuring the same sample and using the same operating procedure over a longer period. In this case, intermediate precision for TPC and TFC was measured on three different days [42]. From the precision (%CV) of phytochemical compounds, the repeatability and intermediate precision values for TPC were 1.12 and 1.10, respectively (Table 4). According to validation guidance AOAC, an analyte concentration is 0.1%, and a %CV is 3.7. Therefore, the precision of TPC could meet the requirements. Likewise, the repeatability and intermediate precision values of TFC were 0.67 and 0.71, respectively, as shown in Table 4. According to validation guidance [2], an analyte concentration of 1% and a %CV of 2.7% were selected as satisfactory values [2]. Hence, the precision (%CV) of TFC could meet the satisfactory requirements. Therefore, it was concluded that the first extraction cycle had a recovery value of more than 90%, and the precision (%CV) values were lower than 5% for both repeatability and intermediate precision. Hence, the developed UAE extraction method was considered precise and accurate.

Table 4. The method validation of UAE extraction by considering precision (%CV) and %recovery on each cycle of extraction for TPC and TFC

Phytochemical Parameters	Repeatability precision (%CV)		%Recovery		
	Repeatability	Intermediate precision	Cycle 1	Cycle 2	Cycle 3
TPC	1.12	1.10	96.33 ± 0.55	3.67 ± 0.55	0
TFC	0.67	0.71	94.29 ± 0.24	5.42 ± 0.14	0.29 ± 0.10

Analysis of chlorogenic acid by HPLC-DAD: The extract from the optimized conditions was further analyzed for the phenolic compound. As a result, the calibration curve constructed from the chlorogenic acid standard was linear throughout the concentration range of 0.5-100 µg mL^{-1}. The chlorogenic acid peak was observed in the retention time at 28.08 min. The coefficient of determination (R^2) was consistently obtained at 0.9964, confirming the robustness and reliability of the analytical method within the studied concentration range. The results of the chlorogenic acid calibration curve are shown in Table 5.

Table 5. Regression equation of chlorogenic acid standard curve by HPLC-DAD

Compound	Retention time (min)	Regression equation	R^2
Chlorogenic acid	28.08	y = 24.028x – 238.04	0.9964

Twenty-five µg mL^{-1} of the chlorogenic acid standard was spiked into ethanol extracts of *E. elatior* inflorescence. It was conducted to confirm the chromatogram of the chlorogenic peak for both Thai and Indonesian *E. elatior* inflorescence. The area under the spiked chlorogenic peak in Thai and Indonesian samples could increase 1.4 times compared to ethanol extracts, as shown in Figure 4 and Figure 5.

E. elatior inflorescence extracted by validated extraction method contains chlorogenic acid. *E. elatior* Thailand had 4.31±0.43 mg g^{-1} dry matter chlorogenic acid content. *E. elatior* Indonesia also had 4.45±0.27 mg g^{-1} dry matter chlorogenic acid content (Table 6). The chlorogenic acid content in this study's inflorescence extracts is higher than that reported in a previous study. A previous study [17] reported that the *E. elatior* inflorescence from Malaysia was extracted using the refluxed method with a water solvent. It had 75.79 mg 100 g^{-1} dry matter chlorogenic acid content. Differences in the extraction methods could cause a difference in chlorogenic acid content. *E. elatior* inflorescences from Thailand and Indonesia contained slightly different amounts of chlorogenic acid. This was due to several factors, including the genetic composition of the flower variety and the growing environment [59].

Table 6. Quantitative analysis of chlorogenic acid from Thai *E. elatior* inflorescence, compared to Indonesian *E. elatior* inflorescence using the HPLC technique.

Sample	Content of chlorogenic acid (mg g^{-1})
***E. elatior* Thailand**	4.31±0.43
***E. elatior* Indonesia**	4.45±0.27

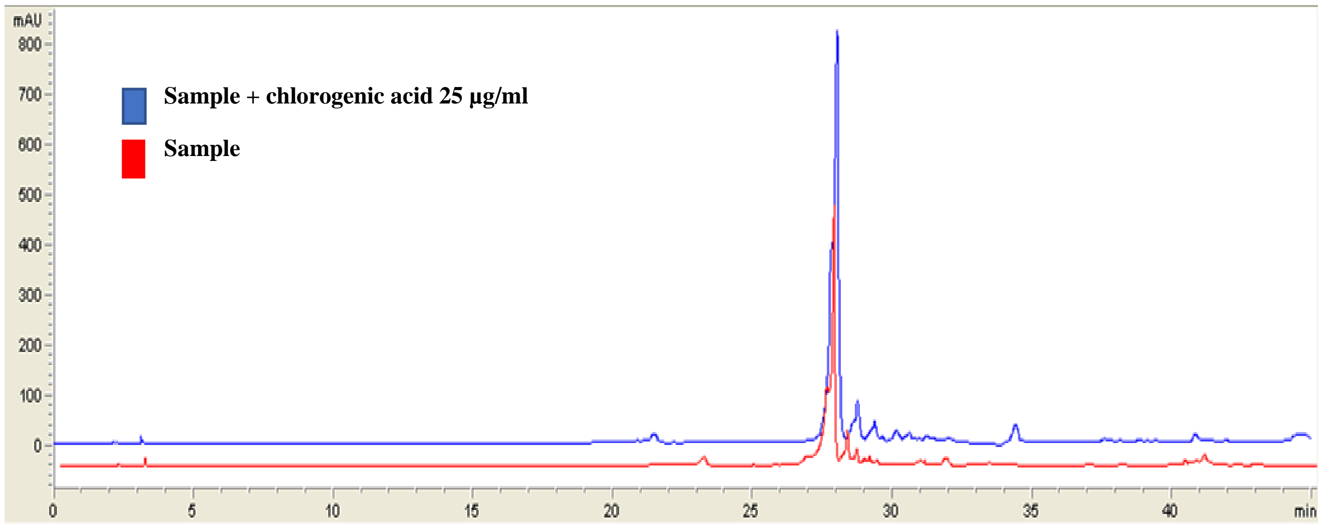


Figure 4. The chromatogram of spiked chlorogenic acid standard at the concentration of 25 µg mL^{-1} was overlayed on *E. elatior* extracts from Thailand at the wavelength λ_{325} nm.

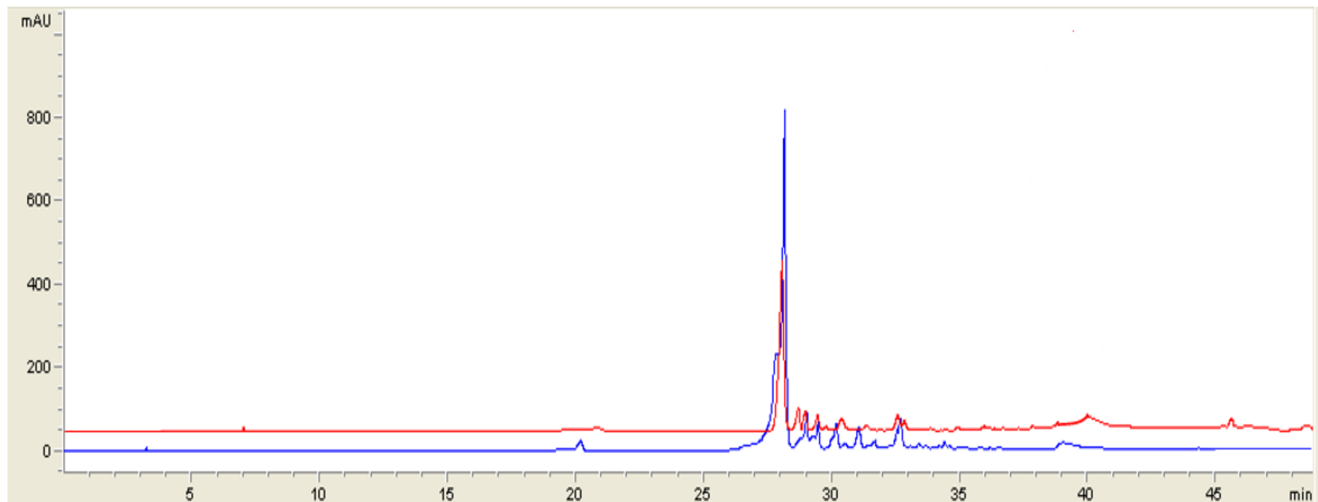


Figure 5. The chromatogram of spiked chlorogenic acid standard at the concentration of 25 µg mL^{-1} was overlayed on *E. elatior* extracts from Indonesia at the wavelength λ_{325} nm.

The application of *E. elatior* tea in the drying methods: According to previous research, thermal processing was performed to preserve and extend the shelf life of edible products. It would affect the polyphenol profile and the antioxidant capacity. The drying process can decrease the water content, inducing degradation, chemical reactions, and enzymatic activities of herbal tea [5]. Therefore, it impacted the preparation of herbal teas by significantly decreasing polyphenol content and antioxidant capacity, reducing tea quality and health benefits [16, 31]. According to the previous research [35] on making herbal tea from Roselle (*Hibiscus sabdariffa* L.) flowers using hot air drying at 80 °C, it could retain total phenolic content and antioxidant capacity. Although the anthocyanin content could decrease during the thermal process, it was observed from their color and functional properties. It was concluded that higher temperatures and drying times are involved in the stability of anthocyanin. The effect of undesirable chemical reactions on food quality could happen when drying was performed at a temperature of ≥50°C [37].

For the development of herbal tea from *E. elatior* inflorescences, the inflorescences were subjected to three drying processes compared to freeze-drying treatment. The drying methods studied included sun drying (32-45 °C), cabinet drying at 50 °C, and shade drying (30-32 °C) for approximately 5 days to reach 12-13% moisture content and compared to freeze drying. The moisture contents of young and mature *E. elatior* inflorescences from Thailand and Indonesia were compared, and their phytochemical and antioxidant activities were evaluated.

Figure 6 shows that the moisture content of young and mature inflorescences from Thailand for cabinet drying was significantly 8.87±0.14% and 7.78±0.67%, respectively. Meanwhile, the inflorescences from Indonesia had a moisture content of 5.94± 0.08% and 5.27±0.09%, respectively, under the same drying condition. When the inflorescences were exposed to sun drying, the moisture content of these inflorescences from Thailand showed a significant increase at 12.83±0.12% and 12.99±0.19%, respectively. On the other hand, those from Indonesia deceased as 11.11±0.39% and 10.31 ±0.71%, respectively. Under the shade drying condition, the moisture contents of young and mature inflorescences from Thailand were significantly provided as 11.01±0.55% and 12.04±0.35%, while those from Indonesia were 12.82 ±0.55% and 10.32±0.13%. Lastly, the moisture contents of those Thai inflorescences for freeze drying were 8.75±0.20% and 8.16±0.24%, whereas those Indonesian inflorescences were 6.69± 0.09% and 7.07±0.12%. The moisture content in the drying process of flowers with cabinet drying was the lowest content among other treatments, similar to the freeze-drying process. This occurred because the drying rate of cabinet drying was accelerated compared to the drying rate of natural drying, such as sun drying and shade drying [29]. The moisture content of sun and shade-dried samples was higher than that of freeze-dried samples. Because of the drying rate of natural drying, it was slower than freeze drying, and the reabsorption of moisture at night might occur in natural drying [15]. The moisture content of fresh *E. elatior* inflorescence in a previous study [41] was between 89-95%. *E. elatior* inflorescences that were already dried had lower moisture content than fresh flowers. The decrease in moisture content was caused by water molecules contained in plant cells evaporating owing to heat during the drying process [25].

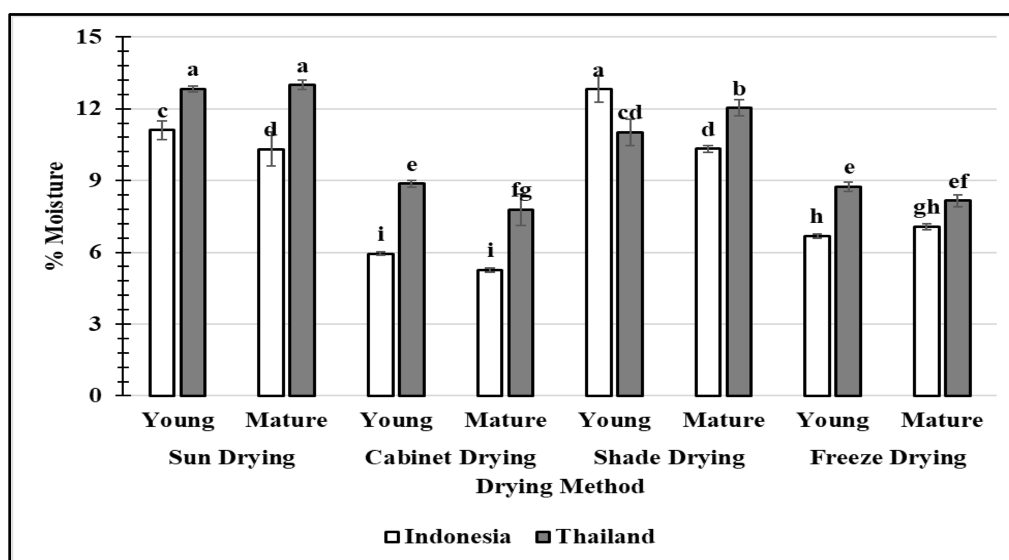


Figure 6. The moisture content of *E. elatior* inflorescences from Indonesia and Thailand on various drying methods: sun drying, cabinet drying, shade drying, and freeze-drying. The standard deviation is displayed as an error bar. A different small letter in different bar graphs means significant differences based on Fisher's Least-Significant Difference (LSD) (p < 0.05).

The total phenolic content (TPC) of young and mature *E. elatior* from Indonesia was 10.44±0.09 mg GAE g^{-1} and 10.89±0.11 mg GAE g^{-1} when subjected to freeze drying. In comparison, the TPC of young and mature *E. elatior* from Thailand under the same conditions was 8.72±0.67 mg GAE g^{-1} and 11.93±0.02 mg GAE g^{-1}. When the *E. elatior* samples from Indonesia were dried using cabinet drying, the TPC values for young and mature plants were 9.71±0.24 mg GAE g^{-1} and 9.66±0.22 mg GAE g^{-1}. On the other hand, the TPC values for young and mature *E. elatior* from Thailand with cabinet drying were 8.20±0.19 mg GAE g^{-1} and 10.81 ±0.21 mg GAE g^{-1}. Sun drying of *E. elatior* from Indonesia resulted in TPC values of 3.97± 0.02 mg GAE g^{-1} for young plants and 4.52±0.14 mg GAE g^{-1} for mature plants. In contrast, sun drying of *E. elatior* from Thailand yielded TPC values of 7.44±0.09 mg GAE g^{-1} for young plants and 4.56±0.23 mg GAE g^{-1} for mature plants. Lastly, when shade drying was employed, the TPC values for young and mature *E. elatior* from Indonesia were 2.810 ± 0.064 mg GAE/g and 6.28±0.11 mg GAE g^{-1}, respectively. Similarly, the TPC values for young and mature *E. elatior* from Thailand with shade drying were 7.63±0.03 mg GAE g^{-1} and 8.58 ± 0.21 mg GAE g^{-1}, as shown in Figure 7. Freeze-drying and cabinet-drying treatments had significantly higher total phenolic than sun-drying and shade-drying. Freeze-drying caused the formation of porosity in plant tissues. The increased porosity that occurs in freeze-drying could increase the extraction efficiency of phenolic compounds. The high phenolic content in freeze-drying could be associated with the mention [39]. Another factor affecting the flower phenolic content was the drying rate. The cabinet drying rate was the highest, but it caused wrinkled petals, which could also increase the extraction of active ingredients from the cells [29].

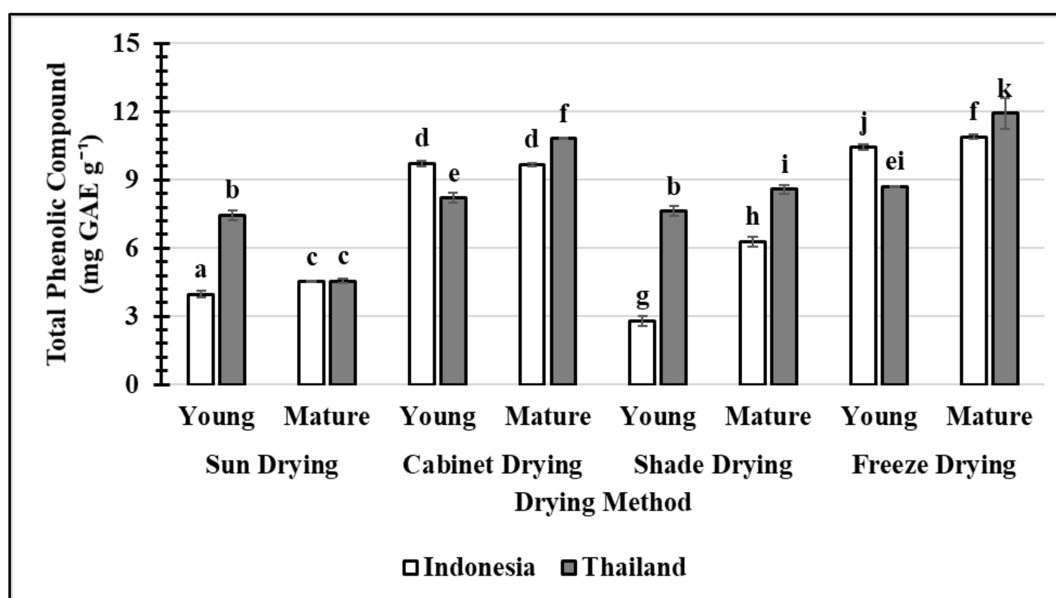


Figure 7. TPCs of *E. elatior* inflorescences from Indonesia and Thailand use various drying methods, such as sun drying, cabinet drying, shade drying, and freeze-drying, to prepare herbal tea. The standard deviation is displayed as an error bar. A different small letter in different bar graphs means significant differences based on Fisher's Least-Significant Difference (LSD) ($p < 0.05$).

According to Figure 8, the total flavonoid content (TFC) of young and mature *E. elatior* from Indonesia subjected to freeze-drying was measured at 14.35±0.81 mg QE g^{-1} and 15.34± 0.50 mg QE g^{-1}. In comparison, the

TFC of young and mature *E. elatior* from Thailand under the same conditions yielded values of 11.65±0.42 mg QE g^{-1} and 12.73±0.86 mg QE g^{-1}, respectively. When the drying method was changed to cabinet drying, the TFC of young and mature *E. elatior* from Indonesia decreased significantly to 1.40±0.65 mg QE g^{-1} and 4.88±0.18 mg QE g^{-1}. At the same time, the samples from Thailand had the values of 1.15±0.18 mg QE g^{-1} and 3.69±0.08 mg QE g^{-1}, respectively. Sun drying of young and mature *E. elatior* from Indonesia resulted in TFC values of 2.33±0.14 mg QE g^{-1} and 1.70±0.05 mg QE g^{-1}, whereas the samples from Thailand showed values of 1.47±0.15 mg QE g^{-1} and 2.30±0.18 mg QE g^{-1}. Finally, shade drying of the inflorescence from Indonesia and Thailand led to TFC values of 1.55±0.30 mg QE g^{-1} 1.62±0.17 mg QE g^{-1}, and 1.24±0.04 mg QE g^{-1}, and 3.64±0.39 mg QEg^{-1}. The drying process with sun, shade, and cabinet drying had a significantly lower total flavonoid value than freeze drying. The primary cause of flavonoid damage was exposure to high temperatures for longer. Besides sensitivity to high temperatures, phenolic and flavonoid compounds were also sensitive to atmospheric environments [47]. The drying caused damage to polyphenols in the sample when the exposure to oxygen, sunlight, and UV light was for a prolonged period [29].

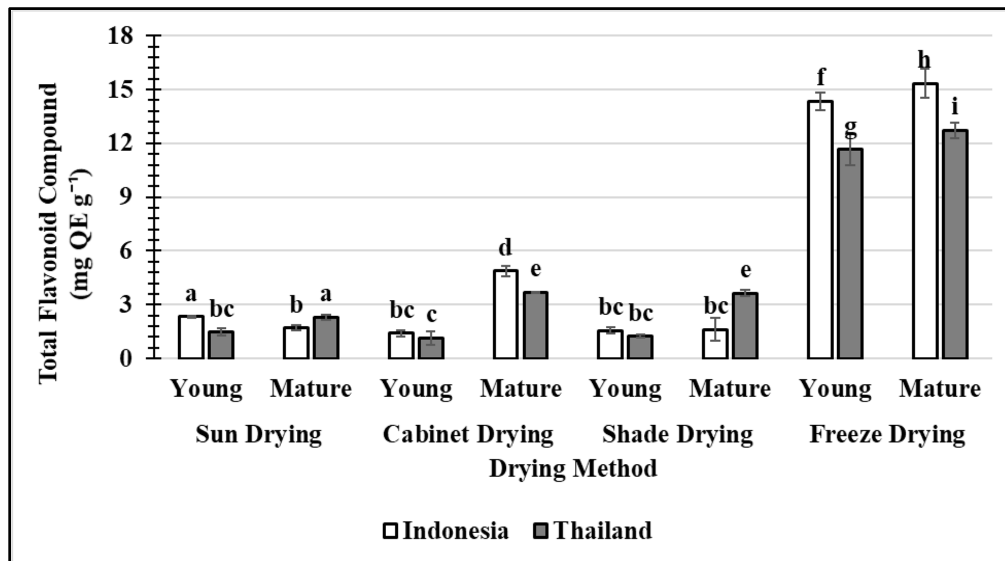


Figure 8. Total flavonoid contents of *E. elatior* inflorescences from Indonesia and Thailand on various drying methods: sun drying, cabinet drying, shade drying, and freeze drying for preparing an herbal tea. The standard deviation is displayed as an error bar. A different small letter in different bar graphs means significant differences based on Fisher's Least-Significant Difference (LSD)($p < 0.05$).

The overall %inhibition of DPPH· scavenging antioxidant activity on dried inflorescences for various drying processes was discussed. According to the obtained results of the DPPH· scavenging antioxidant assay for the different drying processes, except freeze drying, the inflorescences from Indonesia had higher inhibition of DPPH· scavenging antioxidant activity than those from Thailand, as shown in Figure 9. These higher DPPH antioxidant activities were relevant to TPCs (Figure 7) and TFCs (Figure 8) because phenolic and flavonoids exhibit potential antioxidant properties. Therefore, the TFCs from Indonesian inflorescences for sun drying (young), cabinet drying, shade drying (young), and freeze drying were higher than those from Thailand. This caused the %inhibition of DPPH· scavenging antioxidant activity on dried inflorescences from Indonesia to be higher than

that of Thailand, except for freeze drying. However, these factors affect various drying processes, including temperature and chemical reactions, such as the Maillard reaction through antioxidant properties. The temperature might cause an alteration in phenolic and flavonoid compositions. Then, various drying processes substantially affected the inhibition of DPPH˙ scavenging antioxidant activity on the dried inflorescence. Increasing %inhibition of DPPH˙ scavenging antioxidant activity for dried inflorescences might be related to partially oxidized polyphenols, which had been shown to have higher antioxidant activity than unoxidized polyphenols. In addition, the Maillard reaction induced by heat treatment exhibits strong antioxidant properties [1].

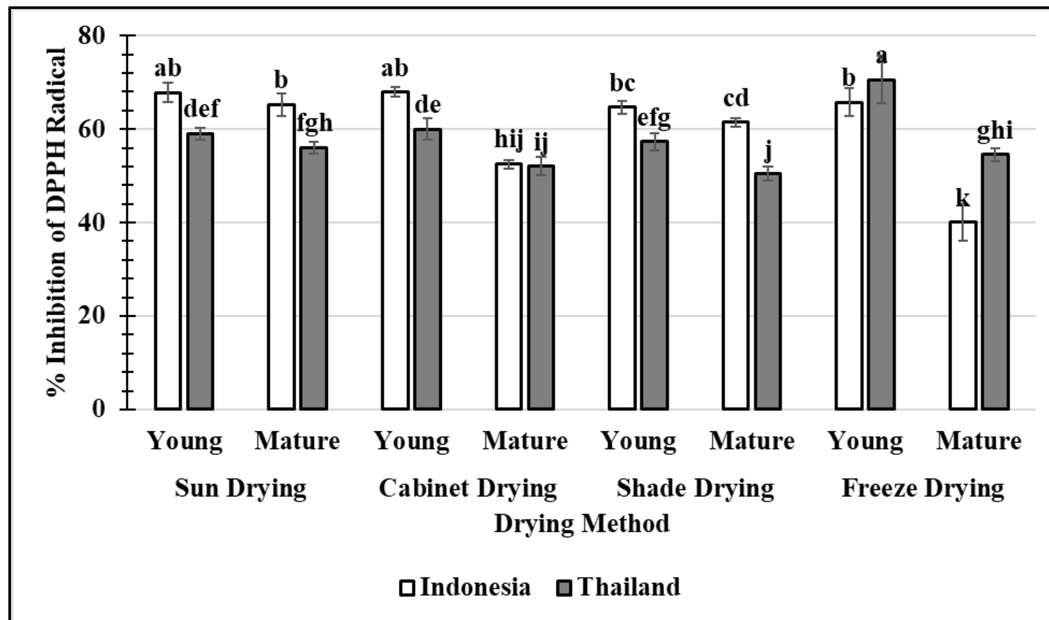


Figure 9. %Inhibition of DPPH˙ scavenging antioxidant activity of *E. elatior* inflorescences from Indonesia and Thailand for four drying processes: sun drying, cabinet drying, shade drying, and freeze-drying treatments. The standard deviation is displayed as an error bar. A different small letter in different bar graphs means significant differences based on Fisher's Least-Significant Difference (LSD) ($p < 0.05$).

The young *E. elatior* inflorescence had significantly higher %inhibition of DPPH• scavenging antioxidant activity than mature *E. elatior* inflorescence. The previous study [60] recommended that different phenolic compounds were the cause of varying %inhibition of antioxidant activities depending on the part of the plant evaluated. Furthermore, previous research [12] suggested that the enhancement of the natural antioxidant properties or the occurrence of new compounds with antioxidant properties might happen during food processing. In addition to the different phenolic and flavonoid types of *E. elatior* inflorescences, ascorbic acid (vitamin C) contributes to antioxidant properties. The previous study [41] reported that *E. elatior* inflorescence had higher vitamin C content than other edible flowers among the ginger family (Zingiberaceae). Vitamin C is an important antioxidant that prevents oxidative stress in plants during photosynthesis. Therefore, vitamin C content was preserved during the freeze-drying process. %Inhibition of DPPH• scavenging antioxidant activity for dried inflorescence from Thailand for freeze-drying treatment

might have been higher than those from Indonesia. At the same time, TPC and TFC from Indonesia were more significant than those from Thailand for the same drying process.

CONCLUSIONS

In summary, the UAE extraction was optimized using BBD and Pareto chart to analyze the influence of solid-to-solvent ratio, solvent concentration, and extraction temperature on the phytochemicals. The method validation showed over 90% recovery and less than 5% precision, indicating high recovery and repeatability precision. The analysis of chlorogenic acid in ethanol extract of *E. elatior* inflorescence from Indonesia and Thailand revealed slight differences due to genetic and environmental factors. Different drying processes for *E. elatior* bracts affected TPCs, TFCs, and DPPH$^{\cdot}$ scavenging activity. Cabinet drying resulted in lower moisture content, higher TPCs, and lower TFCs than freeze-drying. The DPPH scavenging activity was slightly higher in cabinet drying. Indonesia *E. elatior* inflorescences showed higher TPCs, TFCs, and antioxidant activity than those from Thailand. The development of the optimized UAE method using BBD can enhance bioactive compound yields, improving the health benefits of a final product.

List of abbreviations: BBD, Box-Behnken Design; UAE, ultrasound-assisted extraction; TPC, total phenolic content; TFC, total flavonoid content; DPPH, 1,1-Diphenyl-2-picrylhydrazyl; RSM, response surface methodology; CV, coefficient of variations; GAE, gallic acid equivalent; QE, quercetin equivalent; CGA, chlorogenic acid.

Competing interests: The authors declare that they have no competing interests.

Authors' contributions: **Amila Firdhauzi**: Contributed by conducting research in the research design and writing of the original manuscript. Vatcharee Seechamnanturakit: Control and supervision during the research and manuscript review. Widiastuti Setyaningsih: Conceptualized and supervised the research. Chutha Takahashi Yupanqui: Controlled the timing of the research.

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