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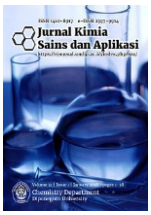

Optimizing Ultrasound-Assisted Extraction Methods of *Etlingera elatior* Using Response Surface Methodology for High Performance Liquid Chromatography Fingerprinting

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Abstract

Kecombrang flower (*Etlingera elatior*) is widely used in traditional medicine and contains various metabolites. High-performance liquid chromatography (HPLC) fingerprinting can be employed as an analytical technique to comprehensively reveal the metabolite profile, while ultrasound-assisted extraction (UAE) was developed to optimize metabolite extraction. This study aims to determine the optimal extraction conditions for *E. elatior* and apply these conditions in HPLC fingerprinting. This study utilized central composite design (CCD) and response surface methodology (RSM) to optimize the extraction of *E. elatior* flowers, focusing on extraction time and the simplicia-to-solvent ratio. The optimal extraction results were applied to HPLC fingerprints of the flowers, leaves, and stems of *E. elatior*. The chromatograms were further analyzed using chemometric methods, namely principal component analysis (PCA), partial least squares discriminant analysis (PLSDA), and hierarchical cluster analysis (HCA) to classify and interpret the variability of metabolite profiles in different parts of *E. elatior*. The optimal UAE conditions were determined to be a time of 46 minutes and a simplicia-to-solvent ratio of 1:25 (g/mL). Chemometric analysis revealed that the samples were well clustered, which reflects the similarity of metabolites among them. The HCA further showed that the metabolite profile of *E. elatior* flowers is closely related to that of the stems.

1. Introduction

Indonesia has significant potential for medicinal plants due to its high level of biodiversity. Medicinal plants are defined as those that produce one or more active compounds used for treatment [1]. One notable medicinal plant in Indonesia is the Kecombrang (*Etlingera elatior*). *E. elatior* is among the most widely utilized plants in Indonesia and offers various health benefits, including antioxidant, anticancer, and antimicrobial activities [2]. The parts of the *E. elatior* plant used for health treatments include the leaves, flowers, fruit, and rhizomes. The flowers of *E. elatior* are commonly used in traditional medicine due to their high content of secondary metabolites, such as phenols, flavonoids, glycosides, saponins, tannins, steroids, and terpenoids [3].

Although *E. elatior* is known to contain numerous active compounds, no publication currently comprehensively identifies all of these compounds. Fingerprint analysis is an analytical method that effectively reveals the metabolic profile of medicinal plants. This analysis can be performed using various instruments, including ultra-high-performance liquid chromatography (UHPLC), high-performance liquid chromatography-mass spectrometry (HPLC-MS), nuclear magnetic resonance (NMR) spectroscopy, UV-Vis spectrophotometry, and HPLC [4, 5]. In this study, HPLC instruments were employed to conduct the non-targeted fingerprint analysis. Previous research has demonstrated that HPLC can effectively analyze the fingerprinting of plants such as *Zingiber officinale* [6], *Kaempferia galanga* [7], and *Curcuma longa* rhizomes [8].

Significant results in fingerprinting profile analysis will be achieved when the metabolite extraction method from the sample is optimized. Extraction is the crucial first step in analyzing herbal plants, particularly to obtain metabolites [9]. Therefore, optimizing the extraction method is essential to maximize metabolite yield and ensure a high-quality fingerprint profile. In this study, the UAE method was selected, utilizing a sonicator for its efficiency, as it requires less time and solvent than other techniques. Factors influencing the UAE include extraction time and the ratio of simplicia to solvent [10], both of which must be controlled to obtain optimal extracts. Prolonged extraction can damage components and reduce bioactivity [11]. Additionally, while increasing the amount of solvent generally enhances the release of target compounds, this effect plateaus after reaching a certain solvent volume [12].

The optimization of plant extraction methods can be achieved through experimental design using a chemometric approach. Chemometrics involves the application of mathematical and statistical methods to collect and analyze multivariate data [13]. In this approach, a common method for optimizing extraction is combining central composite design (CCD) and response surface methodology (RSM). CCD is utilized to develop a model for the extraction optimization experimental design. At the same time, RSM analyzes the relationship between independent variables and the response, thereby optimizing the extraction process of *E. elatior* flowers based on the CCD model [14]. The RSM-CCD technique has been effectively used to achieve optimal extraction results in various plants, such as *Daucus carota* [15], *Opuntia engelmannii* cultivar (cv.) valencia [16], and *Dipsacus asperoides* [17].

There are various metabolites in *E. elatior,* making it essential to develop extraction methods to maximize their yield. Optimizing the UAE method for *E. elatior* is crucial, and it can then be applied to HPLC fingerprints for further study. Previous studies on *E. elatior* often lacked systematic optimization of extraction parameters, leading to suboptimal metabolite recovery. Additionally, most fingerprinting methods employed limited analytical tools and did not incorporate chemometric analysis, which made it challenging to interpret complex metabolite profiles. These limitations underscore the need for optimized extraction methods and advanced data analysis to achieve comprehensive and reproducible phytochemical characterization of *E. elatior*. While *E. elatior* is increasingly recognized as a medicinal plant, its extraction process has rarely been optimized using advanced experimental methods.

This study uniquely combines UAE with RSM to improve the extraction conditions for *E. elatior*, enhancing both efficiency and reproducibility. Furthermore, the integration of HPLC fingerprinting with chemometric analysis offers a novel approach to differentiate metabolite profiles across various plant parts, facilitating standardization. Overall, this study establishes a comprehensive framework for optimizing the extraction and profiling of *E. elatior*, with significant potential for quality control in herbal materials.

2. Experimental

This research was conducted in an experimental laboratory using samples of *E. elatior* flowers. Data were obtained by optimizing the extraction of some parts of *E. elatior* with water as a solvent through the UAE technique using a sonicator (Power Sonic 405, Hwashin Technology Corporation, Korea). The response was measured using HPLC instrumentation, and the data were subsequently analyzed using RSM. The optimized extraction conditions were then applied to HPLC fingerprinting in combination with chemometrics.

2.1. Preparation of the Extract

The flowers, stems, and leaves of *E. elatior* were collected from the Banyumas district. The plant materials were cleaned with tap water, and a portion was dried in a hot air oven at 50°C. The dried flowers, stems, and leaves were ground and stored in sealed containers protected from light until used as simplicia. UAE was employed to extract metabolites from *E. elatior*. One gram of simplicia was weighed and transferred into an Erlenmeyer flask, which was sonicated at 60 kHz and 30°C. Deionized water from OneLab (Jakarta, Indonesia) served as the solvent for extraction. The sonication time and the ratio of simplicia to solvent were determined using a CCD model, as shown in Table 1. After the UAE extraction process, the filtrate was collected and filtered through a 0.45 µm nylon membrane syringe filter before being injected into the HPLC.

Table 1. Experimental conditions of CCD

Run	Coded value		Actual value	
	x_1	x_2	Time; x_1 (minute)	Simplicia to solvent ratio; x_2 (g:mL)
1	-α	0	10	1:30
2	0	0	35	1:30
3	-1	+1	17	1:44
4	-1	-1	17	1:16
5	+1	-1	53	1:16
6	+α	0	60	1:30
7	0	+α	35	1:50
8	0	0	35	1:30
9	0	-α	35	1:10
10	0	0	35	1:30
11	0	0	35	1:30
12	+1	+1	53	1:44
13	0	0	35	1:30

Table 2. Gradient elution program for HPLC mobile phase

Time (minute)	Mobile phase-A Acetonitrile (%)	Mobile phase-B Deionized water (%)
0	20	80
10	20	80
20	80	20
23	80	20
25	20	80
30	20	80

2.2. Chromatographic Conditions

Metabolite analysis of *E. elatior* extract was performed using an Agilent 1220 Infinity II LC system equipped with a UV detector set at 254 nm. Separation was achieved on a C8 column (4.6 × 150 mm) with a gradient elution, where mobile phase A was 100% HPLC-grade acetonitrile (Merck Millipore, Darmstadt, Germany) and mobile phase B was 100% deionized water, as detailed in Table 2. The flow rate was maintained at 1.0 mL/min, with an injection volume of 20 µL [18].

2.3. Optimization of Extraction Condition Evaluation Using Experimental Design

The optimization was carried out using RSM with two independent variables: extraction time (x_1) and the ratio of simplicia to solvent (x_2). Experimental data from the CCD were analyzed using Design Expert software (version 8.0.6, Stat-Ease, Minneapolis, MN, USA), with the total area under the curve (AUC) from the HPLC chromatogram as the response. In this framework, RSM was applied to evaluate the effects of extraction time and solvent ratio on the total peak area, which served as an indicator of extraction efficiency and metabolite richness. The objective was to identify the optimal conditions for maximizing metabolite recovery from *E. elatior*.

2.4. Application of Optimal Extraction Conditions for HPLC Fingerprinting of *E. elatior*

The optimized conditions for UAE from *E. elatior* flowers were subsequently applied to extract metabolites from other parts of the plant, specifically the stem and leaves. HPLC fingerprinting analysis was conducted with three replicate measurements for each sample to compare the metabolite profiles of the samples. The resulting chromatograms were then analyzed using chemometric techniques, including PCA, PLS-DA, and HCA. A quality control (QC) sample was used to evaluate the reliability of replicate measurements and the robustness of the HPLC fingerprinting method. QC samples were prepared by combining equal volumes of each extract and were injected periodically during the HPLC sequence. Consistency in peak intensities and retention times was monitored to assess system stability and analytical repeatability.

Data analysis was conducted using chemometric techniques with MetaboAnalyst software. Median normalization and log transformations were applied to individual values to address data distribution issues. This was followed by auto-scaling, which centers the data and divides each variable by its standard deviation. PCA produces a score plot that groups samples based on their similarity; samples located near each other on the plot indicate a higher model similarity. PLS-DA creates a score plot that clearly shows the separation between different groups. HCA generates a dendrogram that forms clusters, with the vertical scale indicating the differences between them. A smaller distance between two clusters on this scale signifies greater similarity.

3. Results and Discussion

3.1. Optimization of Extraction Methods

HPLC was used to analyze the *E. elatior* flower extract through 13 combinations of CCD experimental runs. This study utilized a non-targeted HPLC fingerprinting approach, which necessitated the optimization of HPLC conditions in preliminary experiments. The selected conditions were based on chromatographic profiles that displayed several well-resolved peaks with sufficient intensity, confirming their appropriateness for subsequent chemometric analysis. Figure 1 shows 13 chromatograms obtained from the UAE of *E. elatior* flowers, which serve as the extraction model. The chromatograms were evaluated for retention times ranging from 1.567 minutes to 30 minutes. Retention times of less than 1.567 minutes indicate void volume and were excluded from the analysis. This exclusion was necessary to eliminate the absorption response of the sample solvent and the peak response observed during the column pre-conditioning step.

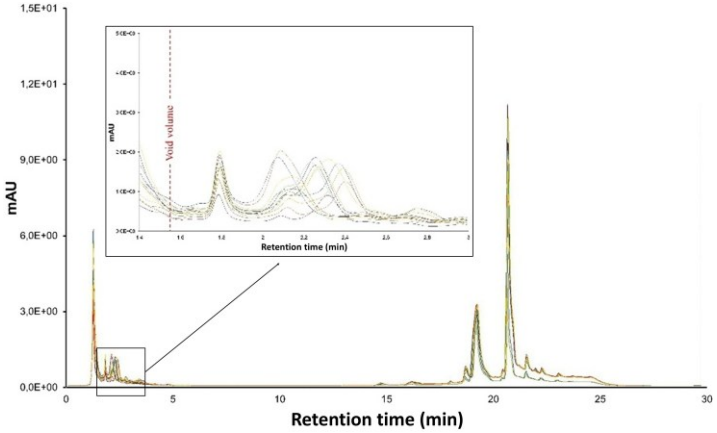

Figure 1. Overlay of 13 chromatograms from the optimization of *E. elatior* flower extraction

Table 3. Results of verification of the CCD-RSM for UAE *E. elatior* flower extract

Extraction time (minutes)	Simplicia-to-solvent ratio (g: mL)	Prediction	Verification	95% PI low	95% PI high
46	1:25	2110.15	1901	1721.53	2382.4
52	1:25	2051.97	187.67	1662.6	2279.27
46	1:34	1966.22	1680.67	1635.12	2297.33

The peak area data for each chromatogram were processed and normalized using the median normalization method. This approach enhances data uniformity by dividing each sample's peak area by its median value. Median normalization is preferred over average normalization, as it yields better results and minimizes the impact of extreme outliers [19]. Subsequently, each chromatogram's total peak area data was analyzed to assess the response using RSM.

The CCD-RSM combination is used to optimize the extraction conditions using the UAE method. The extraction temperature, the number of simplicia samples, and the frequency were kept constant at 30°C, 1 gram, and medium frequency (60 kHz), respectively. The responses obtained by RSM were evaluated for suitability against the regression model of each parameter, as determined using the ANOVA test. The ANOVA model was built to determine the relationship between extraction time (x_1) and simplicia-to-solvent ratio (x_2) with the peak area response. The quadratic regression model is shown in Equation (1).

$$Responses = 1811.4 - 517.33x_1 - 295.92x_2 - 88.95x_1^2 - 177.20x_2^2 + 5.75x_1x_2 \quad (1)$$

ANOVA analysis of the quadratic polynomial model developed for the peak area response variable indicates that the linear effects of x_1 and x_2 are significant ($p < 0.05$), affecting the response. In addition, the quadratic effect significantly ($p < 0.05$) influenced the response. On the other hand, the x_1x_2 interaction effect was not significant ($p > 0.05$) in affecting the response. The p-value of 0.0009 ($p < 0.05$) indicates that the model can influence the response. Meanwhile, the resulting lack of fit value is 0.2066. The lack of fit value of the RSM prediction model can be used when the p-value is greater than 0.05 [14]. R^2 and R^2 adjusted show a good correlation between the predicted and the experimental values (the difference between R^2 and R^2 adjusted <0.1). Following the data obtained, the difference in the value of R^2 (0.8408) and R^2 adjusted (0.7270) has a difference of less than 0.2, indicating a reasonable value [14].

Surface plots and contour plots are utilized to visualize the results of the RSM analysis. The surface plots appear curved due to the significant second-order model. Figure 2 illustrates the impact of extraction time and the ratio of simplicia-to-solvent on the total AUC of *E. elatior* flowers. The results indicate that the optimal conditions occur within an extraction time of 42 to 48 minutes and a simplicia-to-solvent ratio of 1:21 to 1:27 g/mL. Notably, prolonging the extraction time tends to decrease the number of detected metabolites. This finding aligns with previous research indicating that extending extraction time does not significantly increase total metabolites and may even reduce them [20]. Conversely, research by Rifai *et al.* [12] suggests that a higher ratio of simplicia-to-solvent enhances the optimal release of target compounds into the solvent while preventing solvent saturation. However, after increasing the solvent volume beyond a certain point, the yield increase becomes relatively small and tends to plateau.

After conducting experiments with 13 treatments based on the model recommended by CCD, RSM identified several optimal points according to the desired criteria. The software analyzes the model best suited for the response conditions to determine the optimal point for the given response. RSM provided 100 recommendations for optimal conditions, from which three points were selected for verification. The first point was chosen from the 100 suggested optimal conditions, featuring the highest total AUC and desirability values: an extraction time of 46 minutes and a simplicia-to-solvent ratio of 1:25, yielding a total AUC of 2110.5 and a desirability value of 1. The other two points were selected from the red boundary area, or optimal region, on the plot to confirm that the area aligns with the predicted value (Figure 2) [14].

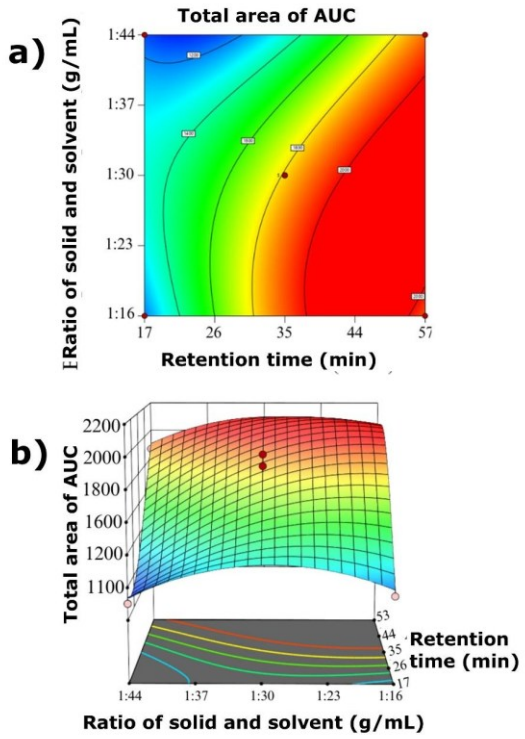

Figure 2. (a) Contour plot and (b) surface plot of the predicted peak area generated from the developed model

The verification result with the highest AUC value was selected at 46 minutes, with a simplicia-to-solvent ratio of 1:25. The verified concentration of the *E. elatior* flower extract was 1901. When compared to the predicted value from the RSM (Table 3), the verification value fell within the established range, between the lower limit (95% PI low) and the upper limit (95% PI high). This indicates that the RSM optimization results are acceptable. These findings suggest that the extraction process used to obtain the optimal results for the *E. elatior* flower extract is consistent.

The prediction interval used in the RSM was set at 95%. This means there is a 95% confidence that the actual outcome of a future experiment, conducted under the same conditions, will fall within the predicted range. This interval takes into account the uncertainty stemming from both the variability of the data and the prediction model itself. Therefore, the validation value aligns well with the predicted value [14]. Notable discrepancies between predicted and verified values often indicate critical issues, such as model overfitting or inadequacy, where the model fits the data well but may not generalize effectively to new conditions.

3.2. High Performance Liquid Chromatography Fingerprinting Application

The optimized extraction results of the *E. elatior* flower extract can be utilized, particularly in HPLC fingerprinting. The samples analyzed included the flowers, stems, and leaves of *E. elatior*. The stems and leaves were included to compare their metabolite profiles to those of the flowers. Each part of the plant was extracted under the optimal UAE conditions, with three replications for accuracy. A small-scale HPLC fingerprinting analysis was conducted to apply the optimized extraction method results using a limited number of samples [11].

Figure 3 displays nine HPLC chromatograms of *E. elatior* samples recorded at 254 nm, showing clear differences in peak shapes and intensities. These variations reflect the unique chemical fingerprints of each sample. Fingerprint analysis aims to identify similarities or differences in the chromatogram profiles between samples based on the identified AUC characteristics. According to the other research, fingerprint analysis typically requires approximately 10- 12 replications for each sample [21]. Therefore, future studies on HPLC fingerprinting of *E. elatior* flowers, stems, and leaves should consider increasing the number of replications.

Figure 3 displays variability in HPLC chromatograms across *E. elatior* tissues, particularly in peak number, intensity, and retention time, reflecting differences in metabolite composition. Notably, flower samples exhibited richer and more diverse profiles, characterized by larger total peak areas and more distinct peaks, indicating a higher metabolite content. In contrast, leaf chromatograms displayed fewer and weaker peaks. These results highlight the importance of using HPLC fingerprinting in conjunction with chemometrics to analyze the chemical complexity of plant tissues.

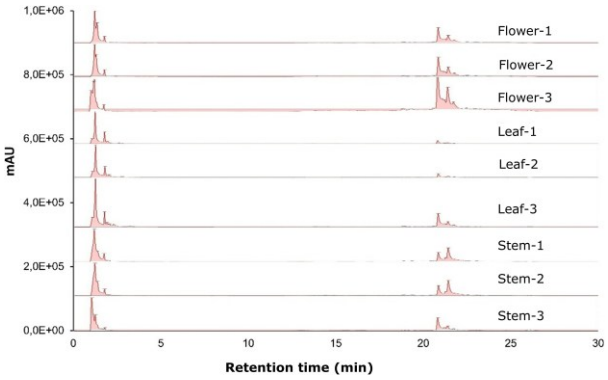

Figure 3. HPLC fingerprinting chromatogram overlay of stem, flower, and leaf samples, each with three replications

The chromatogram data obtained from the fingerprint analysis were processed using chemometrics. A total of 21 peaks were selected from the chromatogram for chemometric analysis, each exhibiting intensities at least three times higher than the baseline noise. This threshold guaranteed a reliable and meaningful representation of the peaks. The chemometric methods employed included PCA, PLS-DA, and HCA. PCA, in particular, is used to differentiate samples through pattern recognition. In this study, PCA was used to analyze and identify the chromatogram patterns of each sample based on their peak area values. The distribution pattern generated from this data provides a qualitative evaluation of the similarities and differences among the samples [13]. The grouping pattern of the fingerprint samples from the PCA analysis is illustrated in the scores plot shown in Figure 4a.

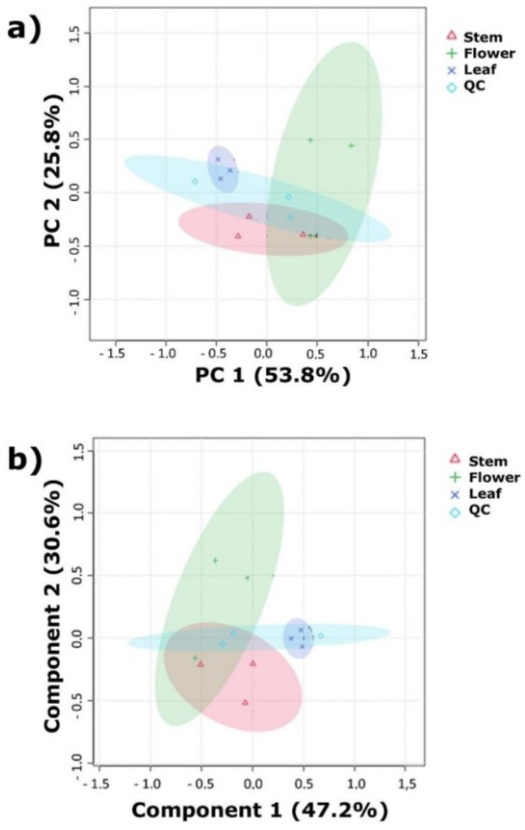

Figure 4. Score plot of (a) PCA HPLC fingerprints and (b) PLS-DA HPLC fingerprints

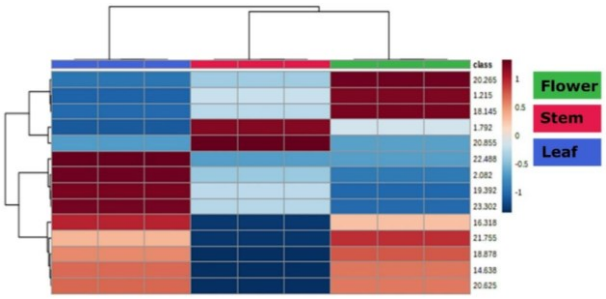

Figure 5. Dendrogram heat maps of HPLC fingerprints for *E. elatior* water extract

Based on Figure 4a, the score plot is made using PC 1 and PC 2 values, which can represent a variance of 79.6% (PC 1 = 53.8% and PC 2 = 25.8%). The first two PCs are used because they are the largest PCs that can group data properly. The closer one cluster is to another, the greater the chemical profile similarity of the samples [13]. The distribution pattern of the flower samples seems closer to the stem samples than the leaf samples.

HPLC fingerprints were analyzed using PLS-DA, an extension of PCA, to confirm distribution patterns and maximize sample differences. PLS-DA was shown to cluster data more effectively than PCA in HPLC fingerprint analysis [22]. As illustrated in Figure 4b, flowers are closer to stems than leaves. Components 1 and 2 account for a variance of 77.7% (with Component 1 at 47.2% and Component 2 at 30.6%), indicating that both components represent the data more effectively.

After conducting PCA and PLS-DA analysis, HCA was applied to evaluate the similarities and differences in chromatogram profiles across samples based on their AUC values. Samples with closely related metabolite profiles are more likely to cluster within the same group or class [22]. The dendrogram from the cluster analysis revealed three major groups based on the similarities among the variables (Figure 5). The HCA results showed that flower samples were closely related to stem samples, consistent with the PCA and PLS-DA findings. Moreover, HCA effectively highlighted the separation of metabolites among stems, flowers, and leaves, a distinction that was less apparent in the PCA and PLS-DA analyses.

PCA, PLS-DA, and HCA results indicate that the metabolites in *E. elatior* flowers are more similar to those in the stems. The compound kaempferol-3-O-glucoside, found in both the flowers and stems of *E. elatior* [23]. Additionally, the essential oil content was analyzed in the flowers, stems, and leaves of *E. elatior*, with the highest concentration found in the flowers, followed by the stems and leaves [24]. This supports the finding that the metabolite content, particularly the essential oil from *E. elatior*, is more similar in the flowers and stems than in the leaves. This is consistent with other research [25] evaluating the similarity among the flowers, stems, and leaves of *Rumex usambarensis*, where the HCA dendrogram demonstrated a close metabolite profile between the flowers and stems.

4. Conclusion

The CCD-RSM experimental design was used to determine the optimal extraction method for UAE extracts of *E. elatior*, resulting in an extraction time of 46 minutes and a simplicia-to-solvent ratio of 1:25. HPLC fingerprinting analysis, combined with chemometric methods, was employed to analyze the profiles of *E. elatior* flowers, stems, and leaves, revealing distinct clustering for each sample. The results from PCA and PLS-DA indicated that the different parts of the plant could be grouped accordingly. Notably, the PCA analysis demonstrated that the metabolite profiles of *E. elatior* flowers were more similar to those of the stems than the leaves, a finding further corroborated by HCA analysis. These findings emphasize the effectiveness of combining optimized extraction with chemometric-assisted HPLC fingerprinting as a reliable strategy for quality control and botanical authentication. Additionally, the insights into the phytochemical similarities among different plant parts provide valuable information for future pharmacognostic studies and the use of resources in both traditional and modern herbal applications.

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