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Enhancing the yield of emodin from *Cassia alata* L. leaves using ultrasound-assisted deep eutectic solvent extraction

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ABSTRACT: Emodin is a bioactive compound found in *Cassia alata* leaves, which has several pharmacological effects. However, the current extraction methods for these leaves produce a low yield of emodin. Deep eutectic solvent (DES), with their numerous advantages, could be a strategy to increase the yield of emodin during the extraction process. The objective of this research was to enhance the yield of emodin from the extraction of *Cassia alata* leaves using DES. After evaluating various DES combinations, the selected DES was found to be lactic acid:choline chloride (2:1). To determine optimal extraction conditions, response surface methodology with Box Behnken Design was employed. The results indicate that the highest total anthraquinone content was obtained at extraction temperature of 53°C, extraction time of 19 minutes, and a solid-to-solvent ratio of 1:20 g/mL. Additionally, partial method validation was conducted for the quantification of emodin in *Cassia alata* leaves using LC-UV instrumentation. The validated method employed the following conditions: isocratic mobile phase of 2% acetic acid:methanol (30:70), flow rate of 0.8 mL/min, wavelength of 288 nm, and C-18 column (150 mm x 4.6 mm, 5 µm). The emodin and total anthraquinone content in the *Cassia alata* leaf extract using the selected DES were higher compared to ethanol extract using the same extraction method. In conclusion, the DES solvent (lactic acid:choline chloride in molar ratio 2:1) can be utilized as an alternative solvent in the extraction of *Cassia alata* leaves, which is more effective and efficient in enhancing emodin yield compared to conventional ethanol solvents.

KEYWORDS: *Senna alata*; anthraquinone; response surface methodology; HPLC; green extraction.

1. INTRODUCTION

Empirically, the *Cassia alata* leaves (CAL) are widely used by the Indonesian to treat skin diseases such as ringworm, constipation, and malaria [1-2]. Some of the bioactive compounds in CAL belong to the anthraquinone group. These bioactive compounds include emodin, aloe-emodin, chrysophanic acid, chrysophanol, isochrysophanol, rhein, physcion, 4,5-dihydroxy-1-hydroxymethylanthrone, and 4,5-dihydroxy-2-hydroxymethylanthraquinone [3]. Among these anthraquinone compounds, the ones with the highest percentage in the methanol extract of CAL are emodin (0.71%) and aloe-emodin (0.42%) [4]. Compared to other parts of the tree, such as the roots, the leaves have three times higher emodin content [5]. Previous studies have revealed that emodin has pharmacological activities as anticancer, anti-inflammatory, antiviral, antibacterial, antiepileptic, antidiabetic, and hepatoprotective agent [6-12].

To enhance the emodin content in the extraction of CAL, specific strategies are required. One such strategy involves applying hydrolysis treatment to the powdered sample. Hydrolysis in the pretreatment process can be performed by adding an acidic substance (such as hydrochloric acid) to produce compounds in their aglycone form [13]. This is due to the fact that the anthraquinone compounds in CAL are primarily found as anthraquinone glycosides, especially O-glycosides [14]. Research by [15] on *Rheum emodi* extraction demonstrated that the total anthraquinone content significantly increased when hydrolysis pretreatment was applied, compared to samples without acid hydrolysis treatment.

Besides incorporating acid hydrolysis into the powdered sample, another approach to boost the extraction yield of compounds involves employing deep eutectic solvent (DES) as the extraction solvent. DES

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consists of a mixture of two or more primary metabolite compounds such as sugar alcohols, organic acids/bases, and amino acids that act as hydrogen bond acceptors (HBA) and hydrogen bond donors (HBD). When these components are combined in precise molar ratios, they create a eutectic point [16]. Due to its classification as a green solvent, utilizing DES as an extraction medium presents various benefits including environmental friendliness, simplified synthesis, enhanced safety, reduced energy consumption, and biodegradability [17]. Past studies have shown that employing DES solvent in combination with ultrasound-assisted extraction (UAE) techniques can elevate the concentration of anthraquinone compounds (including emodin) by about 30% in *Rheum palmatum* compared to traditional chloroform solvent methods [18].

To determine the optimal extraction conditions, one approach that can be commonly used is response surface methodology (RSM). RSM is a statistical and mathematical approach used to find the optimal settings for different variables to achieve the best outcomes in a process or product. It integrates experimental design, regression analysis, and optimization methods to enhance the response of a system [19]. There are several designs in RSM that can be used to optimize extraction conditions, including Box-Behnken Design (BBD), Central Composite Design (CCD), and factorial design [20]. Among these, BBD is advantageous because it is more cost-effective than three-level full factorial designs, especially when dealing with a large number of input factors [21].

As of now, there have been no investigations employing DES as an efficient and green solvent for extracting CAL. Therefore, this study will be the first to examine the selection of DES and optimal extraction conditions to enhance the yield of emodin and total anthraquinone from the extraction of CAL.

2. RESULTS AND DISCUSSION

2.1. Screening DES for extraction

In this study, sample pretreatment was conducted through acid hydrolysis. The addition of HCl breaks the bond of anthraquinone glycosides, resulting in the formation of free anthraquinones. Higher concentrations of added acid lead to increased extraction efficiency. However, excessive addition of acid will generate an excess of protons, disrupting the hydrogen bonds between anthraquinones and DES, thereby reducing extraction efficiency. Therefore, in this study, an optimal HCl concentration of 8% was utilized based on previous research [22].

Five different combinations of DES (Table 1) were used, and one DES that gave the highest levels of emodin and total anthraquinones was selected. The LA:Glu; 1,2-Pd:ChCl; LA:Men and MA:ChCl was chosen as the test solvent because it has been previously used as an extraction solvent for anthraquinone compounds in earlier research [21-22]. Meanwhile, LA:ChCl was selected as the test solvent because of the similarity in the log P of the target compound in previous research to the target compound in this study [23]. The combination of DES LA:ChCl extracted the highest emodin compared to the other four DES ($p<0.05$), yielding 22.835 ± 0.53 µg/g. The next highest emodin levels were obtained with the combinations LA:Glu (12.508 ± 0.12 µg/g), 1,2-Pd:ChCl (11.533 ± 0.20 µg/g), MA:ChCl (6.187 ± 0.04 µg/g), and lastly AL:Men (5.457 ± 0.78 µg/g).

Table 1. Composition of deep eutectic solvents (DESs)

Abbreviation	HBD	HBA		Molar Ratio
LA:Glu	Lactic Acid	Glucose	Water	5:1:3
1,2-Pd:ChCl	1,2-Propanediol	Choline Chloride	Water	2:1:1
LA:Men	Lactic Acid	Menthol	Water	2:1:0
LA:ChCl	Lactic Acid	Choline Chloride	Water	2.65:1.35:1
MA:ChCl	Malic Acid	Choline Chloride	Water	1:1:2

LA: Lactic Acid 1,2-Pd: 1,2 Propanediol Men: Menthol
Glu: Glucose ChCl: Choline Chloride MA: Malic Acid

In addition to emodin levels, the total anthraquinones were also tested. The LA:ChCl combination was also able to extract the most anthraquinone compounds compared to the other four DES combinations ($p<0.05$), yielding 170.646 ± 1.67 µg/g. The next highest total anthraquinone levels were obtained with the combinations 1,2-Pd:ChCl (157.596 ± 3.16 µg/g), LA:Glu (42.240 ± 0.93 µg/g), MA:ChCl (39.408 ± 0.98 µg/g), and lastly LA:Men (37.931 ± 4.16 µg/g). The comparison of emodin and total anthraquinone levels obtained from the five DES combinations used can be seen in Figure 1.

DES LA:ChCl has never been used as an extraction solvent for extracting emodin or other anthraquinone compounds. So far, only two studies have used DES as extraction solvents targeting anthraquinone compounds [21-22]. From these two studies, LA:Glu has been proven to extract the highest emodin levels from

both *Rheum palmatum* and Cassiae semen. LA:ChCl has been used as an extraction solvent in *Rosmarinus officinalis*, targeting rosmarinic acid as the extraction compound [23].

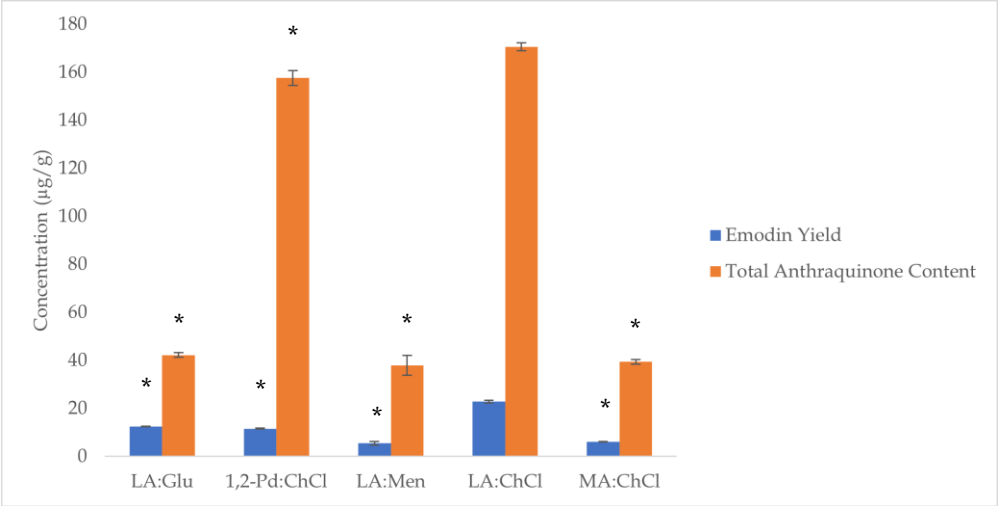

Figure 1. Emodin and total anthraquinones content from the extract DES of CAL. Results that are significantly different from LA:ChCl are marked with an asterisk (*) ($p<0.05$).

The solubility of compounds in an extraction solvent is an important factor in choosing the type of extraction solvent [24]. Considering the LogP values, emodin and rosmarinic acid have similar values. Emodin has LogP of 1.9, while rosmarinic acid has a LogP of 1.8. This is one of the factors contributing to the ability of LA:ChCl to optimally extract emodin as effectively as LA:ChCl extracts rosmarinic acid. This also supports previous research, indicating that in the extraction of compounds using DES, LogP is a key factor influencing extraction efficiency [25]. It was also mentioned that compounds with more polar properties will interact more extensively with more hydrophilic DES, and vice versa.

2.2. LC-UV Validation Method for Emodin Quantification

In this study, method for emodin quantification from CAL was developed based on previous research [26], with modifications to the wavelength and flow rate. The optimal wavelength for detecting the standard emodin compound was determined to be 288 nm. This finding is consistent with other studies that indicate emodin can be detected using a UV-Visible detector at wavelengths of 252, 254, 288, and 437 nm [27]. The previous research [26] did not present the validation results for LOD (Limit of Detection) and LOQ (Limit of Quantification). Compared to previous research that also used an isocratic HPLC system [28], the validated method in this study provides lower LOD, LOQ, and precision values, resulting in better sensitivity. Overall, the validation results of the analytical method in this study (which include parameters such as linearity, LOD, LOQ, precision, and accuracy) meet the requirements of the ICH Q2 (R1) Guidelines and can be used for the quantification of emodin from CAL.

Emodin was detected at retention time 17.59 min with resolution (Rs) value about 1.8. A peak is considered to have good resolution if its value is ≥ 2.0 (ICH Q2 (R1) guideline). One way to improve peak resolution is to develop the elution system from isocratic to gradient [29]. Figure 2. showed chromatographic profile of emodin standard and sample extracts. The chromatogram profiles of CAL extracted using DES and 96% ethanol show similar profiles.

Linearity was performed by measuring the peak area of the standard emodin compound at a series of concentrations: 0.08, 0.3125, 1.25, 2.5, and 5 µg/mL. The emodin calibration curve is shown in Figure 3., with the linear regression equation being $y = 78372x + 6026.1$ ($R^2 = 0.9999$).

Based on the calculations, the LOD and LOQ values were found to be 0.0567 µg/mL and 0.1891 µg/mL, respectively. Precision was evaluated by analyzing the standard emodin compound at a mid-range concentration of 2.5 µg/mL six times consecutively for both intraday and interday variations. All of percentage relative standard deviation (%RSD) values were below 2.0%, meeting the ICH Q2 (R1) Guideline requirements. The validation results for LOD, LOQ and precision are presented in Table 2.

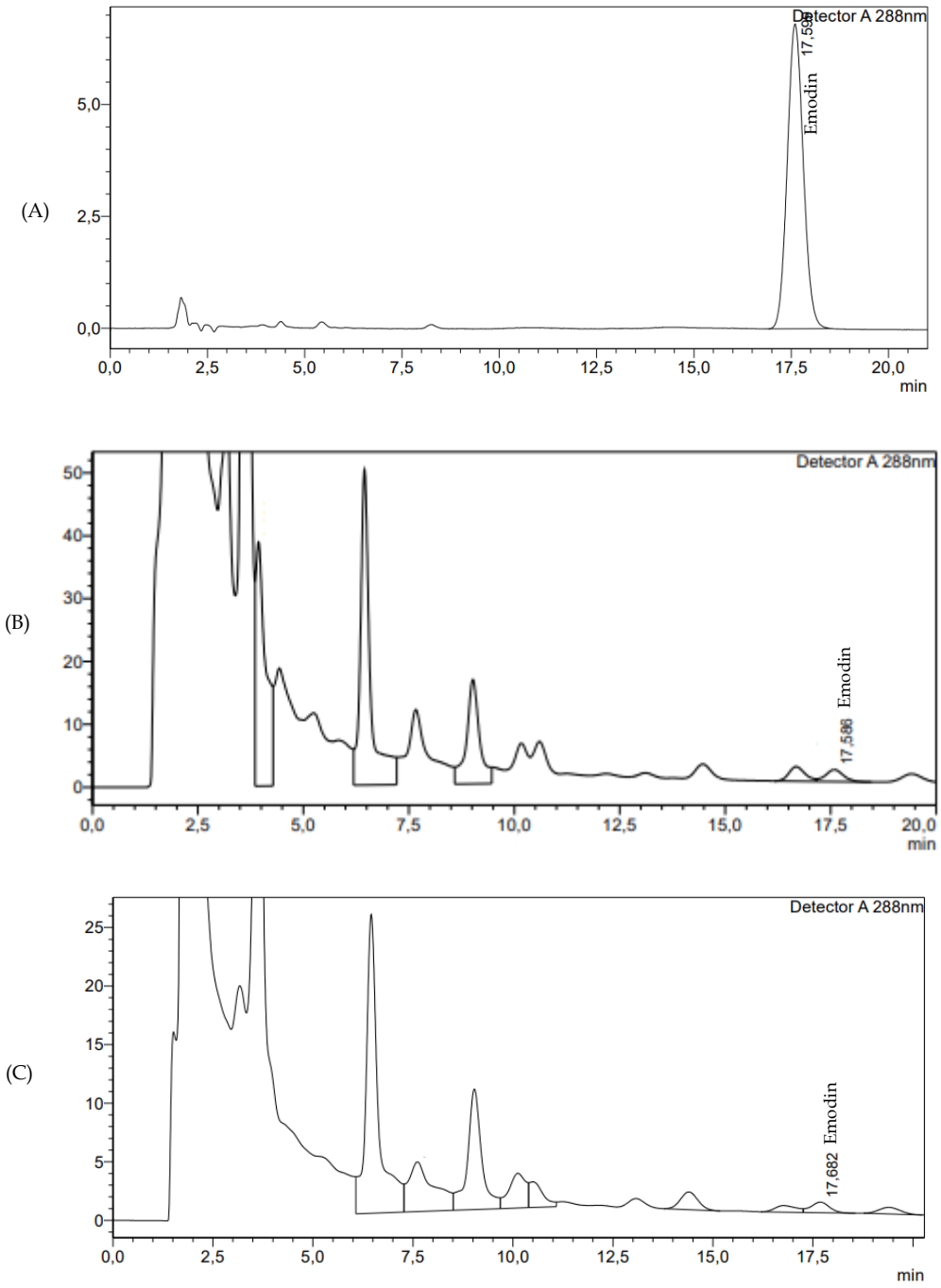

Figure 2. Chromatogram of (A) emodin standard; (B) CAL extract using DES; and (C) CAL extract using 96% ethanol

Accuracy was assessed at three different concentration levels: 2, 6, and 15 µg/mL, each with three replicates. The accuracy data can be found in Table 3. The results showed that the % recovery values ranged from 91.361 ± 0.160 to 104.098 ± 0.646, indicating that the method has good accuracy and comply with ICH Q2 (R1) Guideline requirements.

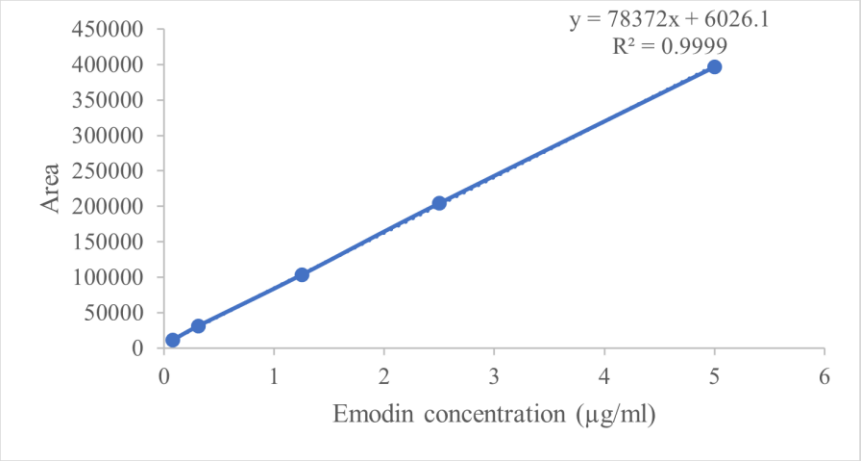

Figure 3. Emodin calibration curve

Table 2. Method validation parameters

Parameters	Results
Regression equation	y = 78372x + 6026.1
Correlation coefficient (r^2)	0.9999
Linear range (µg/mL)	0.08 – 5
LOD (µg/mL)	0.0567
LOQ (µg/mL)	0.1891
Intraday (%RSD)	0.544
Interday (%RSD)	1.549

Table 3. Recovery studies

Theoretical (µg/mL)	Found (µg/mL)	Recovery (%)
2	1.827 ± 0.003	91.361 ± 0.160
6	5.706 ± 0.101	95.106 ± 1.677
15	15.615 ± 0.097	104.098 ± 0.646

2.3. Optimal Condition for UAE using selected DES

The extract of CAL with the selected DES was optimized for its extraction conditions using the statistical method of Response Surface Methodology (RSM) in the form of Box-Behnken Design (BBD). A total of 15 test runs were conducted to verify the prediction model used, and the results can be seen in Table 4.

The highest total anthraquinone content was obtained from sample 11 (169.391 µg/g), with the extraction conditions as follows: temperature of 50°C, time of 20 minutes, and a solid-to-solvent ratio of 1:20 g/mL. Using Design-Expert 13 software, the relationship between factors and responses can also be analyzed, as shown in the three-dimensional (3D) graph in Figure 4. Both in Figure 4(A), 4(B), and 4(C), similar profiles are shown, where at the middle level of each factor, the highest total anthraquinone content is obtained. This suggests that as the levels of each factor increase, the total anthraquinone content can also increase. Nevertheless, at a certain level point, the total anthraquinone content has reached its maximum value, so further increasing the factor levels cannot enhance the obtained total anthraquinone content.

The regression equation used to predict the obtained total anthraquinone content is:

$$y = 164.78 + 18.32x_1 + 1.75x_2 - 4.58x_3 - 23x_1x_2 + 17.05x_1x_3 - 10.94x_2x_3 - 35.67x_1^2 - 28.20x_2^2 - 42.44x_3^2$$

Table 4. Results of BBD of various independent UAE conditions and corresponding responses for total anthraquinone content

Run	Temperature (°C)	Time (min)	Solvent:Solid Ratio (mL/g)	Total Anthraquinone (µg/g)
1	40	20	10	88.627 ± 2.61
2	60	20	30	118.809 ± 0.78
3	50	20	20	164.609 ± 2.35
4	50	10	10	89.602 ± 0.92
5	50	10	30	93.166 ± 0.52
6	50	30	30	76.818 ± 0.39
7	60	10	20	144.730 ± 0.26
8	40	10	20	55.633 ± 0.65
9	60	30	20	100.199 ± 1.05
10	40	30	20	103.088 ± 1.57
11	50	20	20	169.391 ± 0.13
12	50	30	10	117.005 ± 0.20
13	50	20	20	160.341 ± 2.09
14	60	20	10	84.728 ± 1.31
15	40	20	30	54.518 ± 2.75

The results of ANOVA on the prediction model are presented in Table 5. The R^2 value obtained from the software for the prediction model is 0.9845, suggesting that the model can account for 98.45% of the variation. The lack of fit value showed p-value >0.05, indicating that the failure of this prediction model is not significant. In addition, the Predicted R^2 of 0.7816 was in reasonable agreement with the Adjusted R^2 of 0.9567, where, according to the software used, the difference between these values met the requirement of being less than 0.2 In Table 5, the p-values for B-Time and C-Solvent:solid Ratio were larger than 0.05, indicating that time and solvent:solid ratio factors (when considered individually without interaction with other factors) did not contribute significantly to the model. However, the terms AB, AC, BC, A^2, B^2, and C^2 were found to be significant, meaning these terms had a real impact on the measured response and were not due to chance.

2.4. Verification of the model through optimization

From the experimental design used, RSM suggested the optimal extraction conditions at temperature, extraction and solvent:solid ratio of 53°C, 19.1 minutes, of 1:20 g/mL, respectively. Based on these suggestions, the total anthraquinone content was predicted to reach 167.320 (µg/g), as shown in Table 6.

From Table 6, it can be observed that the actual total anthraquinone content was slightly lower than what was predicted by RSM. The difference in results with this slight margin aligns with the high desirability value of 0.982. Desirability value approaching 1 indicates that the recommended extraction conditions can represent the proximity of the actual response to the RSM prediction.

2.5. Comparison of CAL extraction using DES versus ethanol.

Extraction using 96% ethanol refers to previous research [28], under extraction conditions of 60°C for 18 minutes with a solid-to-solvent ratio of 1:25. The comparison results of emodin and total anthraquinone levels in CAL extraction using the DES solvent LA:ChCl and conventional solvent 96% ethanol are displayed in Figure 5. In the ethanol extract, the levels of emodin and total anthraquinone were only 44.189 ± 2.95 µg/g and 34.114 ± 0.85 µg/g, respectively. Meanwhile, in the DES LA:ChCl CAL extract, the levels of emodin and total anthraquinone were 55.911 ± 0.56 µg/g and 110.075 ± 5.64 µg/g, respectively.

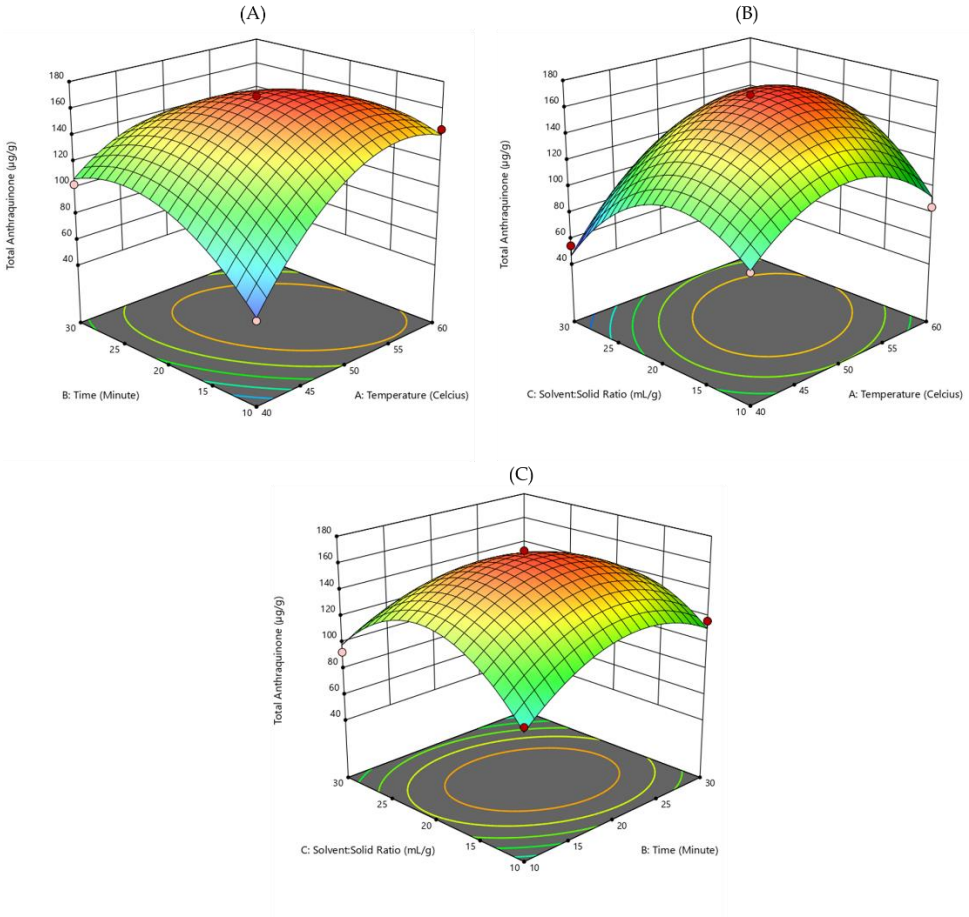

Figure 4. 3D response surface plot of total anthraquinones content versus (A) temperature and time; (B) temperature and solvent:solid ratio; (C) time and solvent:solid ratio

Table 5. ANOVA of prediction model RSM

Source	Sum of Squares	Degree of freedom	Mean Square	F-value	p-value
Model	19095.20	9	2121.69	35.37	0.0005
A-Temperature	2686.38	1	2686.38	44.78	0.0011
B-Time	24.43	1	24.43	0.4072	0.5515
C-Solvent:solid Ratio	167.91	1	167.91	2.80	0.1552
AB	2115.34	1	2115.34	35.26	0.0019
AC	1162.44	1	1162.44	19.38	0.0070
BC	478.55	1	478.55	7.98	0.0369
A^2	4698.59	1	4698.59	78.32	0.0003
B^2	2935.26	1	2935.26	48.93	0.0009
C^2	6649.55	1	6649.55	110.84	0.0001
Residual	299.97	5	59.99		
Lack of Fit	258.97	3	86.32	4.21	0.1978
Pure Error	40.99	2	20.50		
Cor Total	19395.17	14			

Table 6. The optimal extraction conditions for extracting total anthraquinone content (RSM recommendation vs. actual)

	Temperature (°C)	Time (min)	Solvent:Solid Ratio (mL/g)	Total Anthraquinone (µg/g)	Desirability
Recommendation	52.894	19.093	20.156	167.320	0.982
Actual	53	19	20	161.569 ± 6.67	
Error rate (%)				-3.437	

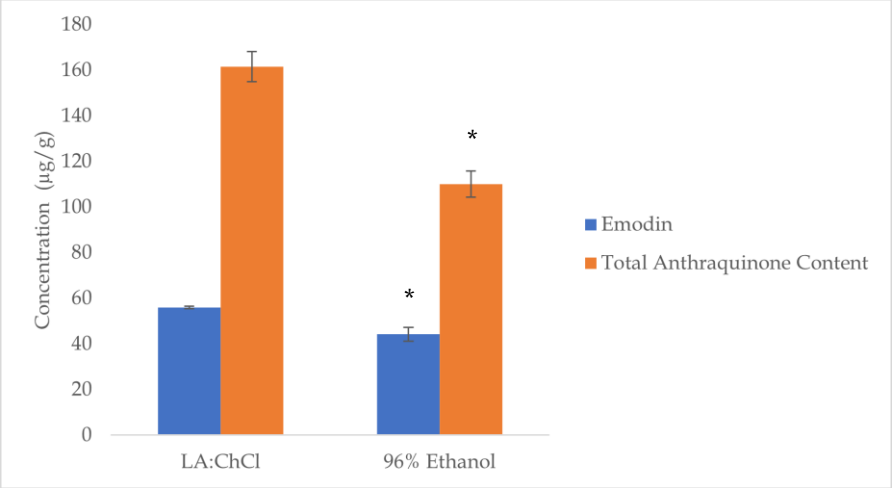

Figure 5. Emodin and total anthraquinone content in the extract of CAL using the solvent LA:ChCl and 96% ethanol. Results showing significant differences compared to LA:ChCl are marked with an asterisk (*) ($p<0.05$).

The DES LA:ChCl is capable of increasing the levels of emodin and total anthraquinones during the extraction process compared to the 96% ethanol. This could be due to the polarity properties of the components composing the HBA and HBD in DES, which can influence the types of compounds extracted. This aligns with the advantages of DES, particularly its increased selectivity in extracting plant compounds [30]. Unlike organic solvents such as ethanol, DES has the unique capability to disrupt plant cell walls, thereby increasing permeability and improving extraction efficiency [31]. However, further research is required to observe the molecular mechanisms of DES (in this case, LA:ChCl) in enhancing extraction yield.

3. CONCLUSION

The DES solvent with the composition of LA:ChCl has been successfully developed as an extraction solvent for CAL, which can enhance the levels of emodin and total anthraquinones compared to 96% ethanol. An analysis method using LC-UV for quantifying emodin in CAL has also been successfully developed. The suggested optimal extraction conditions are a temperature of 53°C, time of 19 minutes, and a solid-to-solvent ratio of 1:20 g/mL. LA:ChCl in a molar ratio of 2:1 can be utilized as an alternative solvent in the extraction of CAL, which is more effective and efficient.

4. MATERIALS AND METHODS

4.1. Chemicals

The chemicals used in this study included emodin, lactic acid (Sigma Aldrich, USA); choline chloride (Shaanxi Qin Health, China); 1,2 propanediol (SK picglobal Co., Ltd., South Korea); glucose, ethanol, methanol, hydrochloric acid (Merck, Germany); menthol crystal (Anhui Fengle Perfume Co., Ltd., China); malic acid (Fuso Chemical Co., Ltd., Japan); ether (Mallinckrodt Pharmaceuticals, United Kingdom).

4.2. Preparation of samples and sample pretreatment

In this work, the dried plant materials were collected and authenticated from UPT Laboratorium Materia Media Batu, Malang, East Java, Indonesia. The dried leaves were grounded using a blender and then sifted through mesh sizes 40 and 80 to achieve powdered of consistent fineness and uniformity. The powdered sample (1000 mg) was hydrolyzed with 25 mL of 8% HCl and heated using a water bath at 90°C for 1 hour. The sample was then cooled to room temperature and mixed with the solvent to be used for extraction.

4.3. Preparation of DES

Each DES component was combined in the specified molar ratios and heated using a magnetic stirrer at 80°C, stirred at 1500 rpm until a clear liquid formed. The DES components used are listed in Table 1.

4.4. Ultrasound-assisted Extraction Procedure

For DESs screening step, 0.5 grams of the pre-treatment sample were extracted with 10 mL of DES solvent. UAE for 20 minutes at 50°C. The mixture was then centrifuged at 1500 rpm for 15 minutes to separate the powder and supernatant. The powder was discarded, and the supernatant was adjusted to final volume of 10 mL using a volumetric flask.

For comparison, ethanol extraction followed the conditions outlined in previous research [28], performed at 60°C for 18 minutes with a powder-to-solvent ratio of 1:25.

4.5. LC-UV Validation Method for Emodin Quantification

Emodin quantification in the CAL extract was conducted using an isocratic LC-UV method. Partial method validation was performed including parameters of linearity, sensitivity, accuracy, and precision. The analysis utilized the Shimadzu LC-20AT HPLC system with a Shimadzu SPD-20A detector. Before analysis, both the samples and the mobile phase were filtered through a 0.45 µm membrane filter. The HPLC analysis conditions are detailed in Table 7.

4.6. Design of experiment (DOE)

In this study, the Box-Behnken Design (BBD) in Design Expert software (version 13, Stat-Ease Inc, free trial, downloaded March 10, 2024) was used to determine the optimal extraction conditions for extracting CAL with the previously selected DES solvent. The response was total anthraquinone content, with extraction temperature, extraction time and solid-to-solvent ratio as the factors. This experiment consisted of 15 runs and Table 8 shows the variations of conditions tested.

Table 7. Analytical conditions of HPLC for emodin quantification

Parameters	Conditions
Column	Agilent Zorbax Eclipse Plus C18 5µm; 150 x 4.6 mm
Flow rate	0.8 mL/min
Injection volume	20 µL
Mobile phase	2% acetic acid : methanol (30 : 70)
Wavelength	288 nm
Run time	20 min

4.7. Determination of Total Anthraquinone Content

Total anthraquinones were quantified using UV-Visible spectrophotometer with wavelength at 510 nm. Derivatization was carried out using 0.5% w/v magnesium acetate in ethanol. Total anthraquinones content calculated emodin equivalent. This method refers to the ASEAN Herbal Medicine of 1993, also conducted by [32-33] with modifications.

Standard emodin solution was prepared in series of concentrations: 1.56, 3.12, 6.24, 12.48, 24.96 µg/mL. Each series was measured at 12.5 mL, then mixed with 10 mL of 0.5% magnesium acetate, and adjusted to final volume of 25 mL using methanol. The absorbance of each solution was then measured and replicated three times.

The powdered sample was weighed and dissolved in the chosen DES solvent. Extraction conditions was performed according to Table 8. The extracted solution was then centrifuged and filtered. The supernatant was mixed with $FeCl_3$ and 8% HCl, then hydrolyzed with UAE for 30 minutes. After partitioning with ether,

the ether layer was collected, washed, and evaporated. The residue was mixed with 10 mL of 0.5% magnesium acetate, and its absorbance was measured at 510 nm using UV-Visible spectrophotometer. This process was repeated three times.

Table 8. Independent variables and their coded levels

Independent variables	Units	Coded levels		
		(-1)	(0)	(+1)
Temperature	°C	40	50	60
Time	min	10	20	30
Solvent-to-solid ratio	mL/g	10	20	30

4.8. Data Analysis

In the DES screening stage, one-way analysis of variance (ANOVA) test (significance level of $p < 0.05$) was conducted using SPSS software version 23 for Windows (IBM, New York, United States). In the DOE stage, data analysis was performed using Design Expert software version 13, Stat-Ease Inc., Minneapolis, MN, USA. Meanwhile, for comparing the selected DES with ethanol solvent, independent samples T-test analysis was employed using SPSS software.

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Conflict of interest statement: The authors have declared no conflict of interest.

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