

# Paper Ind J Chem

*by Admin Admin*

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**Submission date:** 30-Sep-2021 11:53PM (UTC+0700)

**Submission ID:** 1661711804

**File name:** ed\_MAE\_of\_Total\_Polyphenols\_Content\_from\_Eleutherine\_bulbosa.pdf (242.88K)

**Word count:** 5012

**Character count:** 25077

## Optimizing Natural Deep Eutectic Solvent Citric Acid-Glucose Based Microwave-Assisted Extraction of Total Polyphenols Content from *Eleutherine bulbosa* (Mill.) Bulb

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Received: August 9, 2020

Accepted: December 18, 2020

DOI: 10.22146/ijc.58467

**Abstract:** Application of natural deep eutectic solvent (NADES) citric acid-glucose-based microwave-assisted extraction (MAE) method for total polyphenol content (TPC) enriches the use of *Eleutherine bulbosa* (Mill.) bulb more widely and commercially. Therefore, this study aims to optimize NADES citric acid-glucose-based MAE on the TPC from *E. bulbosa* bulbs using response surface methodology (RSM). In the present study, the dried sample of *E. bulbosa* bulb was extracted using the NADES based MAE. Determination of TPC was conducted using Folin-Ciocalteu reagent and standard gallic acid, then measured using a UV-Vis spectrophotometer at 761 nm. Extraction condition optimization of the NADES based MAE method was performed using RSM with Box Behnken Design (four-factors-three-levels and 29 runs) on Design Expert v12. Based on the results study, the optimum condition extraction on the TPC enrichment was obtained at the NADES ratio (citric acid: glucose) 1:1 g/g; solid-liquid ratio 1:8 g/mL; extraction time of 15 min; and 270 Watt microwave power. The confirmation test and scale-up (50 g samples) were obtained with a  $61.63 \pm 2.23$  mg GA/g sample.

**Keywords:** *Eleutherine bulbosa* Mill; microwave-assisted extraction; natural deep eutectic solvent; response surface methodology; total polyphenols content

### ■ INTRODUCTION

*Eleutherine bulbosa* (*E. bulbosa*), known as Bawang Dayak, is a type of onion that grows wild in Kalimantan's tropical forests that has the potential to be developed and identified compounds that are efficacious [1]. *E. bulbosa* bulbs' chemical content includes tannin, flavonoids, quinones, and triterpenoid compound [2]. Besides, some compounds have been successfully isolated, including anthraquinone, naphthoquinone groups (such as elecanacine, eleutherine, elutherole, eleutherine), and eleuthosides A, B, C [3]. *E. bulbosa* bulbs are known to

overcome various diseases such as cancer/cysts (breast/uterus), prostate, diabetes, gout, hypertension, gastrointestinal disturbances, cholesterol, goiter, bronchitis, stamina, and sexual disorders [4]. *E. bulbosa* bulb has enormous potential as a source of raw materials for natural-based medicines. This plant is abundant and easy to grow on Kalimantan island, but so far, commercial use is limited. Therefore, it is necessary to innovate for increasing the commercial use of this plant through the development of natural products with a green extraction approach.

On the other hand, some studies on *E. bulbosa* have been widely reported, including the effect extraction method of this plant against oral glucose tolerance activity [5], sunscreen activity, and TLC profile [6], heat-assisted extraction of phenolic compound [7], and effect of ethanolic extraction of *E. palmifolia* tuber on blood glucose and insulin level [8]. However, the extraction of target secondary metabolites from *E. bulbosa* using a green solvent approach based on non-conventional extraction methods has not been reported.

The development of non-conventional extraction methods is recently increasing, such as the microwave-assisted extraction method (MAE). This method has advantages over other conventional methods because it is expected to shorten the extraction process, minimize the use of solvents, and save energy use through microwave energy in the irradiation process [9].

The choice of solvent and extraction method are the main factors that must be considered to obtain the extraction efficiency of the target compound from natural products. The utilization of Natural Deep Eutectic Solvent (NADES) has increased in recent years. NADES has many advantages as a solvent, such as requires low cost, chemically inert, adjustable viscosity, readily biodegradable, acceptable toxicity, and sustainability [10-12]. NADES is a type of natural solvent in the form of a deep eutectic solvent, composed of a mixture component of primary metabolites (for example, sugar, amino acids, and organic acids). The right constituent of NADES will form a clear and stable liquid. In contrast, the inappropriate constituent components cause the liquid to be unstable, precipitable, and can return to solid form.

In this study, the selection of the composition of NADES in the form of citric acid and glucose is the right combination where glucose has hydrogen bonding acceptor (HBA) properties. Citric acid has hydrogen bonding donor (HBD) properties. Suppose both materials are fused at a specific temperature, forming a stable solution and ready to be used as a green solvent [13]. On the other hand, these two ingredients are pharmaceutical excipients that are safe for consumption, so it is hoped that the extract obtained can be readily consumed because not containing harmful organic solvents.

The combination of NADES with the non-conventional extraction method effectively extracts the desired target compound and minimizes the compound's undesirable extraction. Several studies have reported the success of NADES as an alternative solvent to replace conventional organic solvents, namely the experiment about extraction of flavonoid from *Radix Scutellariae* [14], extraction of phenol compounds from *Cajanus cajan* leaves [15], and extraction of polyphenols and caffeine from robusta coffee beans [16-17]. The optimization process of various factors that influence non-conventional extraction based on NADES is carried out using response surface methodology (RSM).

RSM is a mathematical and statistical tool that has a crucial role in designing, formulating, developing, and analyzing natural product research, mainly in the extraction method optimization process [18]. RSM has the advantage of studying the interaction between various factors on response [19-20]. In this study, box-Behnken design (BBD) with three-level fractional factorial was used to optimize the extraction condition of total polyphenol content (TPC) enrichment. The BBD is more economical and efficient than other factorial designs due to its ability to select points from a three-level factorial arrangement without center point or factorial points. This design requires fewer points than different methods. The BBD has been used extensively to optimize some extraction conditions such as extraction time, extraction temperature, solvent types, solvent concentration, sample-solvent ratio, etc. [20].

Therefore, this study aims to optimize the condition of the NADES citric-glucose acid-based MAE method for the enrichment of TPC from *E. bulbosa* bulbs using RSM.

## ■ EXPERIMENTAL SECTION

### Materials

The samples of *E. bulbosa* bulb was collected from Samarinda, East Kalimantan, Indonesia. The specimen was authenticated and identified at Laboratory of Dendrology, Faculty of Forestry, Universitas Mulawarman, Samarinda, Indonesia. The fresh bulb was washed and cut into small pieces and dried using a dryer

cabinet at 50–60 °C. The citric acid (100% pure food grade non-GMO) and glucose (food grade) were obtained from CV. Chlorogreen, Bandung, Indonesia. Gallic acid (analytical purity), Folin-Ciocalteu reagent, and sodium carbonate were purchased from Merck, Germany (through Elokarsa LLC, Indonesia). The quality test of the chemical purity was carried out before the experiment to ensure that the chemical quality was up to the specifications.

## Procedure

### The procedure of NADES based MAE method

The extraction process was carried out using NADES based MAE by referring to some previous studies [16,21-22], with some modifications adjusting the extraction conditions. Firstly, the composition of NADES (citric acid and glucose) was melted at 50–80 °C until forming a concentrated solution. The NADES solution slowly added distilled water in a ratio of 1:1, 3:1, and 5:1 g/g. Next, 5 g of *E. bulbosa* bulbs were mixed with NADES in a round bottom flask. Secondly, the extraction process was conducted using the MAE method, operated under various conditions, as shown in Table 1. The extract solution and the residue were separated using a Buchner filter; The extract solution was kept in the refrigerator in a closed container until ready to analyze.

### Determination of total polyphenols content

Determination of total polyphenols content (TPC) was done by spectrophotometry using the Folin-Ciocalteu reagent regarding some literature [23-24], with slight modification. Briefly, a 1 mL of sample and standard solution was put into a test tube, added with 5 mL of distilled water and 0.5 mL of Folin-Ciocalteu reagent. After 5 min of incubation, the other reagents such as 2 mL sodium carbonate and 1.5 mL distilled water

was added. Absorbance was measured using a UV-Vis spectrophotometer at 761 nm (in this study, 761 nm was the maximum wavelength) after incubating the mixture of sample for 1 h at room temperature. The TPC in mg GAE/g samples were calculated using a linear regression equation obtained from gallic acid standard solutions at various concentrations (from 12.5 up to 200 µg/mL), namely,  $Y = 0.015 + 0.001559X$ , with an  $R^2$  value of 0.9977, where Y is absorbance, and X is TPC value.

### Design experimental of NADES based MAE optimization

Optimization of the NADES based MAE condition for the TPC enrichment was optimized by response surface methodology (RSM). The optimization process was estimated the interaction between variables and factors (independent parameters) on the TPC value (dependent variable). Box Behnken Design (with four-factor-three-level) was used for experimental design and requiring 29 experiments (with 1 block and 5 center points per block) for optimization extraction condition (in Table 1). A multilinear quadratic regression model was estimated according to the experimental data from different extraction conditions and TPC values using Design Expert v12 software licensed (Statease Inc. Minneapolis, MN, USA).

## RESULTS AND DISCUSSION

The study results from 29 runs obtained extraction conditions with the highest TPC of 85.251 mg GAE/g (NADES ratio of 5:1 g/g, the solid-liquid ratio of 1:12 g/mL, extraction time of 10 min and microwave power of 270 Watt) and the lowest TPC of 27.091 mg GAE/g (NADES ratio 3:1 g/g, a solid-liquid ratio of 1:8, extraction time of 5 min and 270 Watt of microwave power), as shown in Table 2. The determination of TPC

**Table 1.** Experimental design of response surface methodology with box Behnken design using NADES citric acid-glucose as a green solvent

No.	Independent Variables	Unit	Symbol	Factor Levels		
				Low (-1)	Medium (0)	High (+1)
1	NADES (citric acid-glucose) ratio	g/g	$X_1$	1:1	3:1	5:1
2	Liquid-Solid Ratio	mL/g	$X_2$	8:1	10:1	12:1
3	Extraction Time	min	$X_3$	5	10	15
4	Microwave Power	Watts	$X_4$	90	270	450

**Table 2.** Experimental values of TPC of the *E. bulbosa* bulb extract obtained by NADES-MAE at various conditions

Run	NADES Ratio	Liquid-Solid	Extraction Time	Microwave Power	TPC	
	(g/mL) X <sub>1</sub>	ratio (g/g) X <sub>2</sub>	(min) X <sub>3</sub>	(Watt) X <sub>4</sub>	Actual	Predicted
1	3:1 (0)	12:1 (+1)	10 (0)	450 (+1)	38.70	43.37
2	5:1 (+1)	10:1 (0)	5 (-1)	270 (0)	68.42	69.69
3	1:1 (-1)	12:1 (+1)	10 (0)	270 (0)	46.71	43.17
4	3:1 (0)	8:1 (-1)	15 (+1)	270 (0)	38.58	40.39
5	3:1 (0)	10:1 (0)	5 (-1)	450 (+1)	41.93	38.33
6	5:1 (+1)	10:1 (0)	15 (+1)	270 (0)	77.24	77.82
7	3:1 (0)	10:1 (0)	10 (0)	270 (0)	39.77	41.61
8	3:1 (0)	8:1 (-1)	10 (0)	450 (+1)	37.68	35.63
9	3:1 (0)	12:1 (+1)	5 (-1)	270 (0)	35.64	38.86
10	1:1 (-1)	10:1 (0)	15 (+1)	270 (0)	51.13	49.27
11	3:1 (0)	8:1 (-1)	10 (0)	90 (-1)	32.75	27.49
12	3:1 (0)	10:1 (0)	10 (0)	270 (+1)	32.86	41.61
13	3:1 (0)	10:1 (0)	15 (+1)	450 (+1)	41.28	42.62
14	5:1 (+1)	10:1 (0)	10 (0)	450 (+1)	75.67	74.91
15	3:1 (0)	10:1 (0)	10 (0)	270 (0)	49.50	41.61
16	1:1 (-1)	10:1 (0)	10 (0)	90 (-1)	31.28	37.08
17	1:1 (-1)	8:1 (-1)	10 (0)	270 (0)	48.18	48.54
18	3:1 (0)	10:1 (0)	5 (-1)	90 (-1)	31.00	25.21
19	3:1 (0)	12:1 (+1)	10 (0)	90 (-1)	28.22	29.69
20	3:1 (0)	8:1 (-1)	5 (-1)	270 (0)	27.09	33.15
21	3:1 (0)	12:1 (+1)	15 (+1)	270 (0)	45.63	44.62
22	5:1 (+1)	10:1 (0)	10 (0)	10 (-1)	57.54	62.18
23	1:1 (-1)	10:1 (0)	5 (-1)	270 (0)	45.55	44.39
24	5:1 (+1)	8:1 (-1)	10 (0)	270 (0)	66.04	65.12
25	3:1 (0)	10:1 (0)	15 (+1)	90 (-1)	34.77	33.92
26	1:1 (-1)	10:1 (0)	10 (0)	450 (+1)	45.76	46.16
27	5:1 (+1)	12:1 (+1)	10 (0)	270 (0)	85.25	80.25
28	3:1 (0)	10:1 (0)	10 (0)	270 (0)	44.62	41.61
29	3:1 (0)	10:1 (0)	10 (0)	270 (0)	41.31	41.61

in extracts was carried out based on the standard gallic acid obtained. Gallic acid acts as a standard because it is a derivative of hydroxybenzoic acid, which is a simple phenol acid that is pure and stable [16,23-24]. The concentration of gallic acid used was at 12.5 to 200 ppm. Gallic acid analysis was performed using a UV-Vis spectrophotometer, which produced the equation  $Y = 0.015 + 0.001559X$  with a correlation coefficient ( $R^2$ ) of 0.997, where Y is the absorbance. At the same time, the X value is the TPC value. The equation used to calculate

sample levels with various pre-determined factor conditions using RSM.

The absorbance data was measured using a spectrophotometer at 761 nm. The TPC values were calculated using a regression equation formula from a standard calibration curve, then the TPC value obtained in mg GAE/g samples. All data were analyzed using the Design Expert 12 licensed software to determine the optimum conditions for the enrichment of TPC in this study. In Table 3, the standard quadratic model was

suitable for TPC's response with a value of  $p < 0.0001$  ( $< 0.01\%$ ), which shows the probability of a model error of less than 5% or means the quadratic model has a significant effect response to the TPC. Next, an ANOVA analysis was performed on the chosen model. If the value of "prob> F" is lower than 0.05, then the relationship becomes significant, while for the value of "prob> F" higher than 0.1, then the relationship becomes not significant.

The suggested standard quadratic model was based on the insignificant value in the lack of fit with the predicted  $R^2$  value lower than the adjusted  $R^2$  value. However, with a predictive  $R^2$  of 0.7353, this is still in reasonable agreement with the adjusted  $R^2$  of 0.9051. The predicted  $R^2$  and the adjusted  $R^2$  should be within 0.20 of each other. Otherwise, there may be a problem with either the data or the model. The regular  $R^2$  can be artificially inflated by merely adding terms to the model, even if the terms are not statistically significant. The adjusted  $R^2$  plateaus when insignificant terms are added to the model,

and the predicted  $R^2$  will decrease when there are too many insignificant terms. A rule of thumb is that the adjusted and predicted  $R^2$  values should be within 0.2 of each other. For the optimal designs, the mixture polynomials can be reduced before point selection. Reducing the number of coefficients reduces the number of model points required and changes the variable's selection criterion [25-27].

Table 4 shows the best reduced quadratic model based on the results of the analysis of variance. The model F-value of 34.39 and p-value of  $< 0.0001$  less than 0.050 indicate the model terms are significant. There is only a 0.01% chance of an F-value occurring due to interference. In this case,  $X_1$ ,  $X_3$ ,  $X_4$ ,  $X_1X_2$ ,  $X_1^2$ ,  $X_4^2$  are significant model terms. The "Lack of Fit" F-value of 0.47 implies the "Lack of Fit" is not significant relative to the pure error. There is an 87.43% chance that a "Lack of Fit" F-value this large could occur due to noise. Non-significant "Lack of Fit" is right. This value indicates that all four factor have a significant relationship with the

**Table 3.** The selection of mathematical model analysis for optimization

Source	Sequential p-value	Lack of Fit p-value	Adjusted R-Squared	Predicted R-Squared	Recommendation
Linear	0.0082	0.0714	0.3274	0.1163	
Two-Factor Interaction	0.9948	0.0420	0.1335	-0.7595	
Quadratic	$< 0.0001$	0.7209	0.8739	0.7353	Suggested
Cubic	0.8152	0.3899	0.8246	-1.0697	Aliased

**Table 4.** Analysis of variance

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	6016.31	8	752.04	34.39	$< 0.0001$	Significant
$X_1$	2174.97	1	2174.97	99.46	$< 0.0001$	
$X_2$	74.18	1	74.18	3.39	0.0804	
$X_3$	126.69	1	126.69	5.79	0.0259	
$X_4$	357.00	1	357.00	16.33	0.0006	
$X_1X_2$	106.98	1	106.98	4.89	0.0388	
$X_2X_4$	7.69	1	7.69	0.3516	0.5598	
$X_1^2$	2691.64	1	2691.64	123.09	$< 0.0001$	
$X_4^2$	210.73	1	210.73	9.64	0.0056	
Residual	437.36	20	21.87			
Lack of Fit	285.90	16	17.87	0.4719	0.8743	not significant
Pure Error	151.46	4	37.86			
Cor Total	6453.67	28				

response [28-29]. The independent variable of  $X_1$  and  $X_4$  was less than 0.05, indicating statistically significant extraction results. Meanwhile, the independent variables  $X_2$  and  $X_3$  were greater than 0.05, indicating that the variable was not significant to the extraction results. But overall, the interaction between variables influences response.

In Table 5, the coefficient estimates represent the expected change in response per unit change in factor value when all remaining factors are held constant. The intercept in an orthogonal design is the overall average response of all the runs. The coefficients are adjustments around that average based on the factor settings. When the factors are orthogonal, the VIFs are 1; VIFs greater than 1 indicate multi-collinearity. The higher the VIF, the more severe the correlation of factors. As a rough rule, VIFs less than 10 are tolerable.

The equation formula obtained was  $Y = 40.32 + 13.46X_1 + 2.49X_2 + 3.25X_3 + 5.45X_4 + 5.17X_1X_2 + 1.39X_2X_4 + 19.75X_1^2 - 5.90X_4^2$  with  $R^2$  value of 0.9322 with the predicted  $R^2$  of 0.8580 was in reasonable agreement with the adjusted  $R^2$  of 0.9051, the difference was less than 0.2. Adequate precision measures the signal to noise ratio. Greater than four ratios were desirable. The ratio of 21,150 indicates an adequate signal. This model can be used to navigate the design space. In this equation,  $Y$  is the TPC value, where  $X_1$  is the NADES ratio (g/g),  $X_2$  is the solid-liquid ratio (g/mL),  $X_3$  is extraction time (min), and  $D$  is microwave power (Watt). The equation in terms of

actual factors can be used to make predictions about each factor that has given levels' response. Here, the levels should be specified in the original units for each factor. This equation should not determine each factor's relative impact because the coefficients are scaled to accommodate the units of each factor, and the intercept is not at the center of the design space [28-29].

From this equation, the optimum conditions were obtained according to the results of RSM analysis, including 1:1 g/g NADES ratio (citric acid: glucose), 1:8 g/mL solid-liquid ratio, extraction time of 15 min, and 270 Watt microwave power with predicted the TPC of  $51.09 \pm 4.68$  mg GAE/g sample. The confirmation test and scale-up using ten times the number of samples (50 g) were obtained with a  $61.63 \pm 2.23$  mg GAE/g sample, which showed that the TPC produced was within the tolerance interval (TI) range with 95% TI low and 95% TI high. The TI means that the extraction process to obtain TPC's response compared to the extraction conditions predicted by the program is entirely consistent. Each parameter's value is determined by considering the efficiency of the results obtained in the extraction process using time, energy, and solvent consumption.

Based on the above findings, the use of NADES with the composition of citric acid and glucose is more effective than conventional methods. Munaeni et al. reported a TPC of 2.5 mg GAE/g from *E. bulbosa* bulbs extracted by maceration using 96% ethanol [30]. At the

**Table 5.** Coefficient estimate, standard error, confidence interval, and VIF of the reduced quadratic model

Factor	Coefficient Estimate	df	Standard Error	95% Confidence Interval (CI) Low	95% Confidence Interval (CI) High	Variance Inflation Factor (VIF)
Intercept	40.32	1	1.41	37.38	43.27	
$X_1$	13.46	1	1.35	10.65	16.28	1.0000
$X_2$	2.49	1	1.35	-0.3296	5.30	1.0000
$X_3$	3.25	1	1.35	0.4332	6.07	1.0000
$X_4$	5.45	1	1.35	2.64	8.27	1.0000
$X_1X_2$	5.17	1	2.34	0.2942	10.05	1.0000
$X_2X_4$	1.39	1	2.34	-3.49	6.26	1.0000
$X_1^2$	19.75	1	1.78	16.03	23.46	1.02
$X_4^2$	-5.53	1	1.78	-9.24	-1.81	1.02

same time, Shi et al. reported a TPC of 1.4 mg GAE/g of *E. bulbosa* bulbs [34]. Those previous extraction studies show that NADES (citric acid-glucose) combined with the MAE method can be an alternative solvent to replace conventional solvents and extraction methods in certain conditions effectively, quickly, and efficiently environmentally friendly.

## ■ CONCLUSION

The application of the green extraction approach in separating target secondary metabolites from natural products (mainly from plants) continues to experience a significant increase. The use of NADES combined with the MAE method has been successfully optimized using RSM for TPC enrichment. Optimum conditions obtained were 1:1 g/g NADES ratio (citric acid: glucose), 1:8 g/mL solid-liquid ratio, extraction time of 15 min, and 270 Watt microwave power, with the confirmed TPC value of  $61.63 \pm 2.23$  mg GAE/g sample.

## ■ ACKNOWLEDGMENTS

The authors wish to acknowledge the support Rector of Universitas Mulawarman and Head Laboratory of Pharmaceutical Science and Technology, Faculty of Pharmacy, Universitas Mulawarman for providing facilities for the study.

## ■ AUTHOR CONTRIBUTIONS

All author was involved in this study. BY, I.A, SJA, and MA conducted the experiment, He, AI, LR, and FN conducted the DFT calculations, BY, I.A and FN wrote and revised the manuscript. All authors agreed to the final version of this manuscript.

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