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Design of ionic liquid-based microwave-assisted extraction of flavonoids from *Cyclea barbata* Miers. and its lipoxygenase inhibitory activity

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ABSTRACT

Green grass jelly (*Cyclea barbata Miers*.) is a plant from Indonesia that is believed to have anti-inflammatory activity. This study aims to find the optimum condition in grass jelly extraction using the ionic liquid-microwave assisted extraction toward total flavonoid content (TFC) and lipoxygenase activity (LIA). The experimental design was performed using the parameters variable including extraction time, liquid–solid ratio, and ionic liquids concentration to obtain the optimum condition. The optimization analysis used response surface methodology (RSM) with Box–Behnken design (17 trials) to obtain a predictive model with TFC and LIA as a response surface value. In the present study, the optimum condition was suggested by RSM analysis with parameter variables, including extraction time of 17 minutes, 1-butyl-3-methylimidazolium bromide ([BMIM]Br) concentration of 1.76 mol/l, and the liquid–solid ratio of 38.21 ml/g. The equation of regression quadratic model was obtained to predict TFC and LIA as follows: TFC = $2.43A + 2.43B + 1.42C + 0.33AB - 3.20AC - 0.46BC - 4.90A2 - 3.10B^2 - 3.10C^2 + 28.32$ with $R^2 = 0.8336$ and LIA = $0.066A + 8.22B + 0.97C + 2.47AB - 5.86AC + 1.96BC - 9.99A^2 - 13.75B^2 - 13.11C^2 + 63.53$ with $R^2 = 0.9207$, respectively.

INTRODUCTION

Green grass jelly (*Cyclea barbata* Miers.) is a species of Menispermaceae family which is widespread and utilized by people in many parts of Asia such as Indonesia, Thailand, India, and Burma. In Java and Sumatra island of Indonesia, the local community traditionally uses this plant as an anti-inflammatory (Siregar and Miladiyah, 2011). Some research has stated that the green grass jelly leaves contain phenolic compounds (Arkarapanthu *et al*., 2005; Sahasakul *et al*., 2015) flavonoids (flavonol), saponins (Kusmardiyani *et al*., 2014), rich in pectin (Hertanto *et al*., 2017; Yuliarti *et al*., 2017) and nutrients (Sahasakul *et al*., 2015). In general, flavonoids have anti-inflammatory activities as described by Kumar and Pandey (2013).

Inflammation is the body's response to dangerous conditions such as infections and tissue injuries to maintain homeostasis (Medzhitov, 2008). Arachidonic acid metabolism influences inflammatory mechanisms through two pathways: lipoxygenase (LOX) and cyclooxygenase (COX). Both these pathways produce the biological mediators of inflammation including leukotriene, prostaglandin, and thromboxane (Akula and Odhav, 2008).

Conventional extraction methods take a long time and have low efficiency, and also uses excessive organic solvents

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(Azmir *et al.*, 2013; Bubalo *et al.*, 2014; Carda-broch *et al.*, 2008). As the process of extraction develops, many new extraction methods use fewer solvents and consider the efficiency of the extraction time (Azmir *et al.*, 2013; Tang *et al.*, 2012; Wang *et al.*, 2011). Recently, alternative solvents have been found that can replace organic solvents that are low-volatile ionic liquids, capable of dissolving specific compounds, heat-stable, and non-flammable (Chemat *et al.*, 2012). An ionic liquid as a green solvent can be combined with non-conventional extraction methods, mainly using microwave-assisted extraction methods (IL-MAE) (Chemat and Vian, 2014). Some studies have reported that the IL-MAE method successfully extracts secondary metabolite compounds optimally including flavonoid group (Ahmad *et al.*, 2017b; Tang *et al.*, 2013; Wei *et al.*, 2012; Zhang *et al.*, 2014; 2015).

However, the studies related to optimization and application of green extraction using the IL-MAE method and research on inhibition of the lipoxygenase activity from green grass jelly leaves have not been reported. Therefore, this study contributes to the knowledge of the extraction engineering of secondary metabolite and biological properties of this plant. This study aimed to obtain an optimal IL-MAE method to the extraction of flavonoids from green grass jelly leaves and investigate its lipoxygenase inhibitory activity.

MATERIALS AND METHODS

Plant materials

Green grass jelly leaves were obtained from Bogor, West Java, Indonesia and was identified at Laboratory of Biosystematics, Indonesian Institute of Science (Lembaga Ilmu Pengetahuan Indonesia) Cibinong, West Java, Indonesia. The sample specimen (DC-001984-F) was collected at the Laboratory of Pharmacognosy-Phytochemistry, Faculty of Pharmacy, Universitas Indonesia, Depok, West Java, Indonesia.

Chemicals and equipment

Chemicals and equipment used in the study, includes Lipoxygenase kit and linoleate acid, were purchased from Sigma Aldrich. Boric acid, sodium acetate, potassium chloride, sodium hydroxide, and aluminum chloride were bought from Merck, Germany (via PT. Elo Karsa Utama, Indonesia). Aqua DM, ethanol for analysis, methanol for analysis, ethyl acetate for analysis, and ethyl acetate were purchased from PT SmartLab Indonesia, Indonesia. 1-butyl-3-methylimidazolium bromide ([BMIM]Br) was purchased from Shanghai Cheng Jie Chemical Co. LTD, China. Design Expert v10 licensed software (STATE-EASE), spectrophotometer UV-Vis (Jasco V-530, Japan), Modena Microwave 900 Watt (Buono-MV3002), Vortex mixer (Stuart, Germany), Analytical scales (Sartorius 7, Germany), sonicator (Krisbow, Indonesia), micropipette 10–100 µl and 100–1,000 µl (Corning), and pH meter (Eutech Instrument, French).

Ionic liquid-based microwave-assisted extraction procedure

The IL-MAE method was performed based on literature (Ahmad *et al.*, 2017a; 2017b), with a slight modification. Briefly, a 0.5 g of the dried sample of Green grass jelly leaves were extracted using [BMIM]Br solvent in the microwave (Modena 900 W) with some conditions as can be seen in Table 1. The separation of the residue and the extract solution was conducted by filtration. The filtrate (extract) was cooled and left for 10–12 hours at the room temperature. Then, the filtrate was partitioned with liquid–liquid extraction using ethyl acetate. The ethyl acetate layers were separated and evaporated to obtain the dried extracts.

Determination of total flavonoid content

The total flavonoid content (TFC) of each extract was analyzed by aluminum chloride colorimetry method based on literature (Baba and Malik, 2015; Chang *et al.*, 2002; Do *et al.*, 2014) with a slight modification. In brief, 0.5 ml of sample solution or the standard solution were mixed in 1.5 ml methanol, 0.1 ml 10% aluminum chloride, 0.1 ml of 1 M sodium acetate, and 2.8 ml aqua DM (double-distilled water). The mixture was incubated for 30 minutes at the room temperature, and absorbance was measured at 434 nm optimum wavelength. Quercetin solution (3, 4, 5, 6, 7, and 8 µg/ml, respectively) was used as standards.

The yield of TFC in extract sample was examined using equation formula of standards ($Y = 0.0568X + 0.0576$ and $R^2 = 0.9992$, where Y is the yield of TFC and X is the absorbance), and the result was expressed as milligram quercetin equivalent per gram extract (mg QE/g extract).

Lipoxygenase inhibitory activity assay

The lipoxygenase inhibitory activity (LIA) assay was performed according to the study reported by Dzoyem *et al.* (2015). Briefly, a 10-µl extract and baicalein solution were added 1,690 µl of 0.2-M borate buffer solution, 1,000 µl of 300 µM linoleic acid substrate solution, and the mixture was incubated for 10 minutes at 25°C. After the incubation, the mixture was added to 300 µl of 1,000 unit/ml lipoxygenase solution and was incubated again for 15 minutes at 25°C. Then, the sample solution was added to 1,000 µl methanol, and the absorbance was measured at 234 nm using a spectrophotometer UV. Percentage of LIA was examined using equation formula as follows:

$$\% \text{ Inibition} = \frac{(A-B)-(C-D)}{(A-B)} \times 100\%$$

where A is the absorbance of the blank solution with an enzyme; B is the absorbance of blank solution without enzyme; C is the absorbance of the sample solution with an enzyme; and D is the absorbance of sample solution without enzyme.

Optimization of the IL-MAE method by response surface methodology

IL-MAE method optimization was conducted using response surface methodology (RSM) to estimate the interaction of

Table 1. The parameters optimization of the IL-MAE method using RSM with Box–Behnken design.

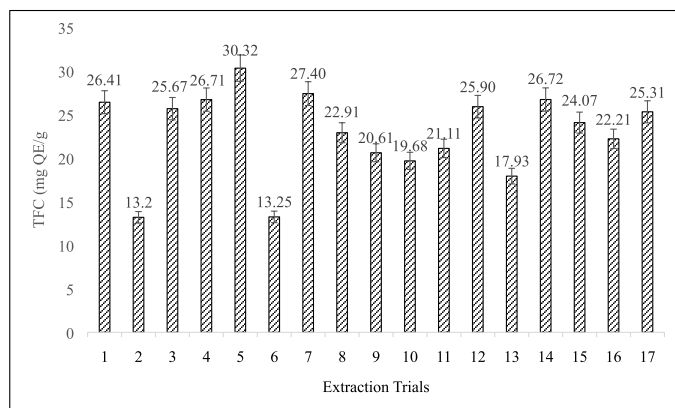| Factors	Unit	Symbol	Range and level		
			−1	0	1
Liquid-solid ratio	ml/g	A	30	40	50
Extraction time	minutes	B	5	15	25
[BMIM]Br concentration	mol/l	C	1	2	3

Figure 1. TFC of each extract using IL-MAE method.

process and factors parameters (independent parameters) against the TFC and LIA (dependent parameters). Box–Behnken design (tree-factor-three level) was used in this study, with 17 trials (in triplicate) for the extraction parameters optimization (Table 1). A regression model was examined with a multilinear quadratic model using Design-Expert v10 software.

RESULTS AND DISCUSSION

Extraction process and pre-optimization

The extraction process was performed using the IL-MAE method to obtain the optimum extraction on total flavonoid content (TFC) and LIA from green grass jelly leaves. Pre-optimization was conducted with some parameters variable condition including extraction time, liquid-solid ratio, and [BMIM]Br concentration to know the best condition. In this study, each parameter's variable was selected (Table 1) based on literature (Fan *et al.*, 2012; Xu *et al.*, 2012).

Total flavonoid content determination

Based on the result of total flavonoid content (TFC) determination (as can be seen in Fig. 1) demonstrated that the maximum of TFC (30.32 mg QE/g) was obtained from fifth trials. On the other hand, the lowest of TFC (13.20 mg QE/g) showed in second trials. Based on different combination of parameter variable at the experimental design, showed extraction time effect of TFC on the range from 5 to 25 minutes performed to obtain the optimal of extraction condition and the optimum of extraction time obtained for 15 minutes extraction time. Regarding liquid–solid ratio, the maximum of TFC was obtained at 40 ml/g with the range from 30 to 50 ml/g the increase of rate (after 40 ml/g) led to a decrease on the TFC. Meanwhile, [BMIM]Br concentration effect of TFC extraction on the range from 1 to 3 mol/l conducted to give the optimal concentration and the optimum concentration obtained at 2 mol/l.

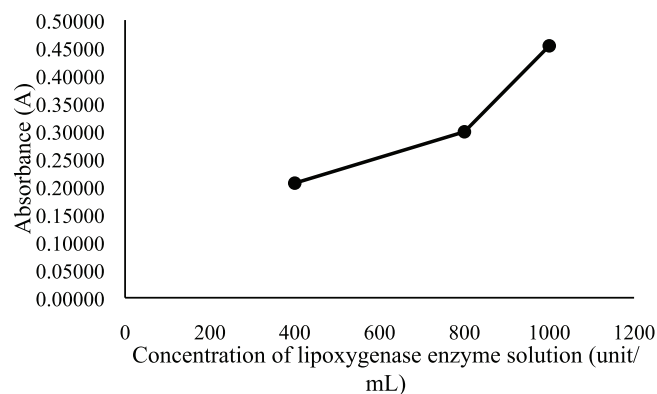### Determination of lipoxygenase inhibitory activity

The preliminary test was performed to obtain the optimum conditions in the LIA assay using some parameters including the pH buffer of the boric acid solution, the linoleic acid substrate, and the lipoxygenase enzyme according to literature (Kumari *et al.*, 2011; Xu *et al.*, 2012). In this study, the
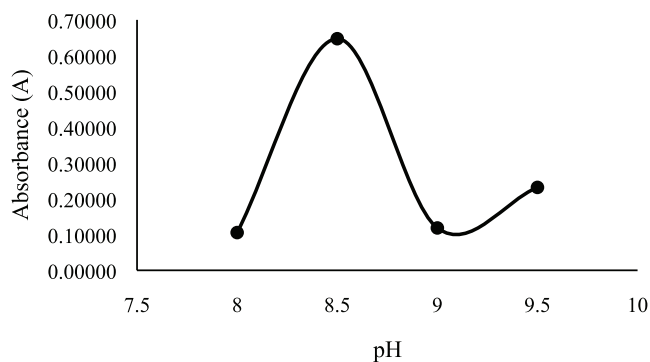
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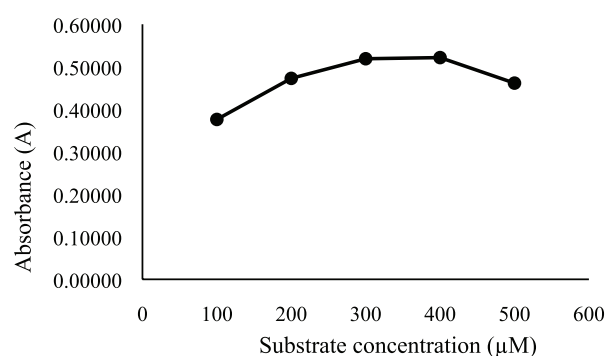
B

C

Figure 2. The optimum conditions of lipoxygenase activity inhibition assay. (A) The pH buffer of the boric acid solution, (B) the linoleic acid substrate, and (C) the lipoxygenase enzyme.

optimization of pH performed on 8.0, 8.5, 9.0, and 9.5 and the optimum condition was obtained at pH 8.5 (Fig. 2A). Optimization of the linoleic acid substrate solution was conducted to determine the optimum substrate concentration, when binding correctly with the enzyme. Based on the previous study, Xu *et al.* (2012) used a 300 µM substrate solution and Kumari *et al.* (2011) used a 250-µM substrate solution. The optimization of the substrate solution

Table 2. Results of lipoxygenase inhibitory activity by baicalein.

Concentration (µg/ml)	Absorbance				(a − b)	(c − d)	% Inhibition	Mean of% Inhibition ± STDEV	Regression equation
	Blank (a)	Blank control (b)	Baicalein (c)	Baicalein control (d)					
0.100	0.8536	0.4435	0.8802	0.6069	0.4101	0.2733	33.36	32.05 ± 2.1642	
			0.8818	0.6080		0.2738	33.23		
			0.8894	0.6005		0.2889	29.55		
0.125	0.8536	0.4435	0.7915	0.5541	0.4101	0.2374	42.12	42.47 ± 0.3172	
			0.7922	0.5567		0.2355	42.56		
			0.7938	0.5589		0.2348	42.73		
0.150	0.8762	0.4650	0.7076	0.4979	0.4112	0.2098	49.00	49.14 ± 0.4974	$y = 0.7864x + 1.8726$
			0.7078	0.4970		0.2108	48.73		$R^2 = 0.9975$
			0.7038	0.4969		0.2069	49.69		$r = 0.9987$
0.200	0.8982	0.4283	0.7157	0.5568	0.4699	0.1589	66.18	65.55 ± 1.1828	
			07123	0.5539		0.1584	66.29		
			0.7134	0.5451		0.1683	64.19		
0.225	0.8982	0.4283	0.5871	0.4594	0.4699	0.1278	72.81	72.07 ± 0.6836	
			0.5906	0.4588		0.1318	71.94		
			0.5927	0.4586		0.1341	71.46		
0.250	0.8650	0.4028	0.5803	0.4870	0.4622	0.0933	79.81	80.24 ± 0.5537	
			0.5747	0.4863		0.0884	80.87		
			0.5802	0.4880		0.0922	80.05		

was done on the concentration of 100, 200, 300, 400, and 500 µM. The optimum condition was 300 µM (Fig. 2B), whereas, the optimization of enzyme solution was performed using several concentrations of 400, 800, and 1,000 unit/ml, and the optimum solution was 1,000 unit/ml (Fig. 2C).

The LIA assay was conducted using baicalein as a standard (Putri *et al.*, 2017). Baicalein is a flavonoid from flavonols groups (Kusmardiyani *et al.*, 2014) that has the capability to inhibit the lipoxygenase activity due to hydroperoxy-octadecadienoate (HPOD) was not formed (Putri *et al.*, 2017). The higher the baicalein content, the less HPOD was formed so that, higher the inhibition percentage (Putri *et al.*, 2017). Table 2 demonstrates that the baicalein concentration from 0.1 to 0.25 µg/ml can inhibit from 33.36% to 80.05% with the linear regression equation of $y = 0.7864x + 1.8526$ ($R^2 = 0.9975$). In this study, the IC_{50} value of baicalein was 0.2 µg/ml which indicates that the enzyme works well. Figure 3 showes the highest of the LIA in 12th trials with an inhibitory percentage of 69.29%. Although the highest levels of LIA were not at fifth trials (where the most top of the TFC) and 12th trials also used the optimum condition variable include 40 ml/g liquid–solid ratio, 15 minutes extraction time, and 2 mol/l [BMIM]Br concentration.

Optimization of the IL-MAE method

Three factors with three levels (−1, 0, +1), including liquid–solid ratio (30, 40, and 50 ml/g), extraction time (5, 15, and 25 minutes), and [BMIM]Br concentration (1, 2, and 3 mol/l) were optimized using RSM with Box–Behnken design. Data analysis was performed to obtain the optimum condition for extraction of flavonoid using the IL-MAE method and LIA, the specific protocols of experimental design of parameters conditions are shown in
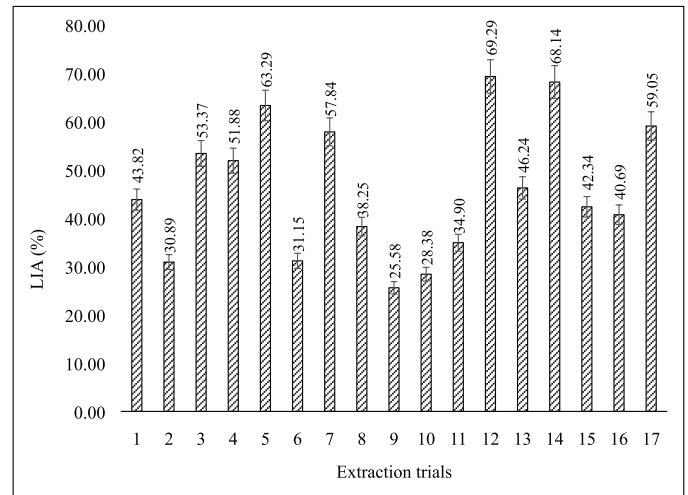
Figure 3. Lipoxygenase inhibitory activity of each extract.

Table 1. Based on the obtained results using RSM analysis with Design Expert v10 software, in Figure 4, demonstrated the three-dimension contour of the response surface for interaction between the factor and process parameters.

To further study the response surface plot for the TFC and LIA of the obtained extracts as a function were analyzed and developed based on the equation formula from RSM analysis results to determine the optimum condition, including liquid–solid ratio to extraction time ([BMIM]Br concentration = 2 mol/l), liquid–solid ratio to [BMIM]Br concentration (extraction time = 15 minutes), and extraction time to [BMIM]Br concentration (liquid-solid ratio = 40 ml/g). The different experimental designs
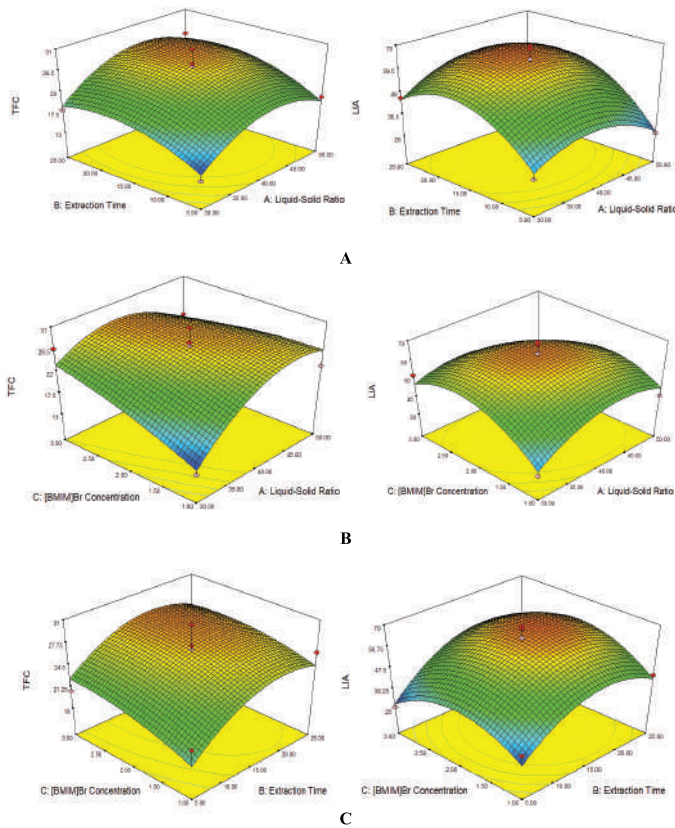
Figure 4. Three dimensional of the contour plot. (A) Interaction of liquid–solid ratio and extraction time. (B) Interaction of liquid–solid ratio and [BMIM]Br concentration. (C) Interaction of extraction time and [BMIM]Br concentration.

Table 3. Experimental design of parameters condition for average TFC and LIA by RSM with Box–Behnken design.

Run/ Trials	Factor A: Liquid-solid Ratio (mL/g)	Factor B: Extraction time (minutes)	Factor C: [BMIM]Br concentration (mol/L)	Total Flavonoid Content (TFC) (mg QE/g)	LIA (%)
1	40 (0)	25 (1)	1 (-1)	26.41	43.82
2	30 (−1)	15 (0)	1 (−1)	13.19	30.89
3	50 (1)	25 (1)	2 (0)	25.67	53.37
4	30 (−1)	15 (0)	3 (1)	26.67	51.88
5	40 (0)	15 (0)	2 (0)	30.32	63.29
6	30 (−1)	5 (−1)	2 (0)	13.25	31.15
7	40 (0)	15 (0)	2 (0)	27.40	57.84
8	50 (1)	15 (0)	3 (1)	22.91	38.25
9	40 (0)	5 (−1)	3 (1)	20.61	25.58
10	50 (1)	5 (−1)	2 (0)	19.68	28.38
11	40 (0)	5 (−1)	1 (−1)	21.11	34.90
12	40 (0)	15 (0)	2 (0)	25.90	69.29
13	30 (−1)	25 (1)	2 (0)	17.93	46.24
14	40 (0)	15 (0)	2 (0)	26.72	68.14
15	40 (0)	25 (1)	3 (1)	24.07	42.34
16	50 (+1)	15 (1)	1 (−1)	22.21	40.69
17	40 (0)	15 (0)	2 (0)	25.31	59.05

of the extraction process with response (TFC and LIA) are demonstrated in Table 3. The obtained data were calculated by multilinear regression analysis based on the response surface. The final model of TFC and LIA from green grass jelly leaf (*C. barbata*) was given by: $TFC = 2.43A + 2.43B + 1.42C + 0.33AB - 3.20AC - 0.46BC - 4.90A^2 - 3.10B^2 - 0.98C^2 + 27.13$ and $LIA = 0.066A + 8.22B + 0.97C + 2.47B - 5.86AC + 1.96BC - 9.99A^2 - 13.75B^2 - 13.11C^2 + 63.53$, respectively.

Table 4 demonstrates the significant model with a *F*-value of 3.90. There is only a 4.33% chance that a "Model *F*-value" this large could occur due to noise. The value of "Prob > *F*" less than 0.0500 indicated model terms were significant.

Table 4. Test of ANOVA significance for regression coefficient by quadratic model with TFC as a response surface value.

Source	Sum of squares	df	Mean Square	F value	p-value Prob > F
Model[a]	309.85	9	34.43	3.90	0.0433
A	47.19	1	47.19	5.34	0.0541
B	47.19	1	47.19	5.34	0.0541
C	16.07	1	16.07	1.82	0.2193
AB	0.43	1	0.43	0.049	0.8319
AC	40.83	1	40.83	4.62	0.0686
BC	0.85	1	0.85	0.096	0.7659
A²	101.15	1	101.15	11.45	0.0117
B²	40.37	1	40.37	4.57	0.0699
C²	4.07	1	4.07	0.46	0.5188
Residual	61.83	7	8.83		
Lack of Fit	46.59	3	15.53	4.08	0.1041
Pure Error	15.24	4	3.81		
Cor Total	371.68	16			

[a]: A model where *A* is the liquid–solid ratio (ml/g), *B* is extraction time (minutes), and *C* is [BMIM]Br concentration.

Table 5. Test of ANOVA significance for regression coefficient by quadratic model with LIA as a response surface value.

Source	Sum of squares	df	Mean square	F value	p-value Prob > F
Model[a]	2,888.09	9	320.90	9.03	0.0042
A	0.035	1	0.035	9.881E-004	0.9758
B	540.55	1	540.55	15.21	0.0059
C	7.51	1	7.51	0.21	0.6597
AB	24.50	1	24.50	0.69	0.4337
AC	137.24	1	137.24	3.86	0.0901
BC	15.37	1	15.37	0.43	0.5318
A²	419.94	1	419.94	11.82	0.0109
B²	796.54	1	769.54	22.42	0.0021
C²	723.87	1	723.87	20.37	0.0028
Residual	248.74	7	35.53		
Lack of Fit	141.98	3	47.33	1.77	0.2911
Pure Error	106.75	4	26.69		
Cor Total	3,136.83	16			

[a]: A model where *A* is the liquid–solid ratio (ml/g), *B* is extraction time (minutes), and *C* is [BMIM]Br concentration.

Table 6. The credibility of the regression equations.

Index mark[a]	Average extraction of TFC	Average extraction of LIA
Std. Dev	2.97	5.96
Mean	22.90	46.18
C.V. (%)	12.98	12.91
PRESS	769.25	2438.52
R-Squared	0.8336	0.9207
Adjust R-Squared	0.6198	0.8188
Predicted R-Squared	−1.0696	0.2226
Adequacy Precision	5.671	7.891

[a]The results were obtained using Design Expert v10.03 software.

The "Lack of Fit F-value" of 4.08 implies the "Lack of Fit" is not significant relative to the pure error. There is a 10.41% chance that a "Lack of Fit F-value" this large could occur due to noise and non-significant "Lack of Fit" is good.

In Table 5, the model F-value of 9.03 implies the model is significant. There is only a 0.42% chance that a "Model F-values" this large could occur due to noise. The value of "Prob > F" less than 0.05 indicate model terms are significant. The significance of the value of 1.77 "Lack of Fit" with F value > 0.05 is not significant relative to the pure error. There is a 29.11% chance that a "Lack of Fit F-value" this large could occur due to noise.

Table 6 shows the credibility of regression equations, the correlation coefficient "R-Squared" of 0.8416 (TFC) and 0.9207 (LIA) were obtained implies this model can express correlation of model more than 80% and 90%, respectively. A negative "Pred R-Squared" of TFC implied that the overall mean might be a better predictor of response than the current model, whereas the "Pred R-Squared" of LIA (0.2226) is not as close to the "Adj R-Squared" of 0.8188 as one might normally expect. "Adequacy Precision" measures the signal-to-noise ratio. The ratio of 5.671 (TFC) and 7.891 (LIA) indicates an adequate signal, where both ratios greater than four was desirable.

Based on the results of RSM analysis, the optimum condition was suggested with the parameter variable as follows: liquid–solid ratio of 38.21 ml/g, extraction time of 17 minutes, and [BMIM]Br concentration of 1.76 mol/l with TFC and LIA prediction of 26.38 ± 1.30 mg QE/g sample and 62.8558% ± 2.62%, respectively.

CONCLUSION

Based on the results, the design of the IL-MAE method to TFC extraction and LIA from Green grass jelly leaf (*C. barbata*) has attempted. The optimum condition of the IL-MAE method was obtained the parameter variables include 17 minutes extraction time, 1.76 mol/l [BMIM]Br concentration, and 38.21 ml/g liquid–solid ratio with the TFC prediction value of 29.07 mg QE/g sample.

This research was an early stage to develop the extraction method to obtain the target compound from natural products plant in an efficient, rapid, and environmental friendly. Future research should be performed for the optimization of extraction procedure in other parameter variables, pilot scale, isolation, and identification of flavonoid content (mainly the active target compound) from this plant by using green chemistry approaches.

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