Optimization of Microwave-assisted Extraction of Anthocyanin from Clitoria Ternatea Flowers

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***Abstract*—Currently, public awareness towards the usage of dyes and colorants from plant extracts as an alternative for synthetic colorants are increasing. Hence, the blue colour of *Clitoria Ternatea* flowers signifies the presence of anthocyanin which has numerous benefits. In this study, microwave-assisted extraction (MAE) of anthocyanin from *Clitoria Ternatea* flowers using ethanol as solvent based on the design of experiment given by response surface methodology was performed. A central composite design was applied to evaluate the optimal conditions of three process variables namely extraction temperature (℃), extraction time (min) and liquid to solid ratio (mL/g). The concentration of anthocyanin was analyzed using pH differential method via UV-Vis Spectrophotometer. At quadratic model of R^2=0.76633, the obtained extraction yield of the blue dye was 49.97%. A validation test was carried out under critical conditions gave 48.61% of extraction yield which was close to the predicted value of 49.36%. As for the total anthocyanin content (TAC), 0.457 mg/g of anthocyanin was obtained. In a nutshell, MAE is a viable extraction method for extracting anthocyanins from *Clitoria Ternatea* flowers. Thus, *Clitoria Ternatea* extract has a potential use as an alternative source of food colorants replacing the existing synthetic dyes.**

***Index Terms*—extraction, microwave-assisted, anthocyanin, optimization, response surface methodology**

I. INTRODUCTION

The roots, seeds and leaves of *Clitoria Ternatea* are utilized for medicinal purposes including enhancing cognitive functions, minimize the effect of dementia, therapy for respiratory issues such as asthma and bronchitis [1]. According to [2], identified that the aerial parts of the plant extracts had numerous benefits on the neurological system of mice. In addition, the flowers are also proven to be a source of anthocyanins and contain other flavonoids which may have enormous medicinal worth particularly as antioxidants [3]. Additionally, it is utilized as a confectionary additive in the food industry as well as pH pointer from nature in the pharmaceutical business [4]. It has the ability to change colour where alkaline solution gives blue colour whereas acidic solution gives red colour [5]. Even though various strong phytoconstituents and high activity natural drugs have been identified from these plants but the problems related to assurance of herbal drugs are still being a challenge for many researchers [6]. Flavonoids are well known for plant secondary metabolites and their biosynthetic pathway has been elucidated in higher plants for instance *Arabidopsis*, maize, and petunia [7]. There are also at least 4000 flavonoids that are not anthocyanins, a large number of which are colorless, white, or pale yellow. Besides, a portion of these do add to the white and cream colours of flower petals (flavones, flavonols) [8]. The solvents commonly used to extract anthocyanins are acidified solutions such as methanol, ethanol, acetone, water, and water mixtures [9]. In the extraction process, the commonly used conventional methods are maceration and Sohxlet extraction. However, organic waste may become an issue and high purity solvents are required in the Soxhlet extraction system which increase the cost [10]. One of the promising and latest extraction technology is using a microwave assisted extraction method. Microwave irradiation has been effectively practiced in many fields including thermal and non-thermal effects which considering the heat up rates, acceleration of ions and also molecular collision [11]. In addition, microwave radiation also has the ability to reduce the processing time and as an energy saving method [12].

A. Microwave-assisted Extraction

Microwave-assisted extraction (MAE) uses microwave energy to promote separation of analytes from the sample matrix into the solvent. Microwave also induces dipole rotation of the molecules which makes electromagnetic to disrupt the hydrogen bonding thereby increases the migration of dissolved ions and assists solvent penetration into the matrix [13]. MAE uses electromagnetic field as a heating mode where the electromagnetic waves penetrate into the samples to provide volumetric heating through ionic conduction and dipole rotation [14].

B. Response Surface Methodology

Response surface methodology (RSM) is utilized for designing statistical experiments, modelling the processes, verifying the statistical significance of the independent variables, and to obtain the optimum operating conditions of the entire process [15]. Thus, being a powerful statistical and mathematical tool, RSM has a major advantage over the one factor at time (OFAT) approach in which it can evaluate the effect of multiple variables

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and their relationships on the response with decreased number of trials [16].

II. Material and Methods

In this study, the samples of *Clitoria Ternatea* flowers were collected around Kuantan, Pahang Sates of Malaysia [17]. 95% laboratory grade ethanol was used as solvent during the extraction process. Acetate buffer (pH 4.5) and potassium chloride buffer (pH 1.0) were used as part of the analysis. An Agilent 7890A-5975 Mass Spectrometry system gas chromatograph (GC) equipped with an Agilent 5975 mass-selective detector was used to determine the composition of the blue dye from *Clitoria Ternatea* flowers.

A. Microwave –Assisted Extraction

Fig. 1 shows the settings of microwave-assisted extraction (MAE). 1.0 gram powder samples of the Clitoria Ternatea flowers were used for the experiment.

Figure 1. Experimental settings of microwave assisted extraction procedure.

B. Response Surface Methodology

Response surface methodology (RSM) was applied to determine the optimum conditions for extraction of anthocyanin from *Clitoria Ternatea*. Total amount of extraction yield was studied using a standard RSM design known as Central Composite Design (CCD). The full quadratic model of response was established using the method of least squares and was expressed as in (1):

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{33}X_3^2 \quad (1)$$

Where: Y: Predicted response βo: Intercept coefficient (offset) β_1, β_2, β_3: Linear terms β_{11}, β_{22}, β_{33}: Quadratic terms β_{12}, β_{13}, β_{23}: Interaction terms X_1, X_2, X_3: Coded independent variables

The model equation was used to predict the optimum value and afterwards to elucidate the interaction between the factors within the specified range [18]. The range of independent variables and experimental design levels variables are shown in Table I.

TABLE I. Experimental Range and Factor Levels of Independent Variables.

Factors	Symbol	Range and Levels				
		-α	-1	0	+1	+α
Temperature (℃)	X_1	32	40	50	60	68
Time (min)	X_2	11	15	20	25	29
Liquid -solid ratio	X_3	11	15	20	25	29

C. pH Differential Method

The blue dye concentration and total anthocyanin content (TAC) were determined by UV-Vis Spectrophotometer. The mass fraction of anthocyanin and Total Anthocyanin Content (TAC) were calculated using formula in (2) [19] and (3), respectively:

$$\text{Anthocyanin (mg/L)} = A \times MW \times DF \times 10^3 / (\varepsilon \times \ell) \quad (2)$$

Where: A= (A520nm – A700nm) pH1.0 - (A520nm – A700nm) pH4.5, Molecular weight, MW= 449.2 g/mol (cyaniding-3-glucoside), DF= dilution factor, ℓ= path length (cm), ε = 26,9000 molar extinction coefficient ($L \times mol^{-1} \times cm^{-1}$), 10^3= factor for conversion from g to mg

TAC (mg/g) = Concentration (mg/L) x Volume of solvent (L) /Sample weight (g) (3)

III. Results and Discussion

Based on Table II, the yield was increased from 32 (run no.1) to 47 % (run no.13) when rising the temperature from 40 to 60 ℃. Also, increasing the temperature from 32 to 50 ℃ enhanced the yield from 30 (run no.4) to 44 % (run no.5). Higher temperature helped to build up intracellular pressure that ruptured the cell walls and resulted in higher solubility of anthocyanin as well as decreased the extract viscosity [14]. Therefore, anthocyanin in *Clitoria Ternatea* was easier to diffuse from cells to solvents at higher temperature and consequently increased the extraction yield. However, the yield was maintained higher at 47 % (run no.3) when the temperature was increased from 60 (run no.13) to 68 ℃ even though the extraction time and liquid to solid ratio were both reduced to 20 min and 25, respectively.

TABLE II. Extraction Yield and Concentration with Different Parameters.

Run No	Temp (℃)	Time (min)	Liquid-solid ratio (g/mL)	Extraction Yield (%)	Conc. mg/L	Conc. mg/g
1	40	25	25	32.18	6.469	0.1617
2	50	11	20	30.05	7.835	0.1567
3	68	20	20	46.64	19.381	0.3876
4	32	20	20	29.99	7.293	0.1459
5	50	20	20	44.45	16.378	0.3276
6	60	25	15	46.54	22.978	0.3447
7	40	15	25	37.65	9.929	0.2482
8	60	15	15	49.97	30.475	0.4571
9	50	20	29	44.72	11.409	0.3309
10	50	29	20	46.68	20.667	0.4133
11	40	15	15	38.33	17.384	0.2608
12	50	20	11	44.42	25.609	0.2817
13	60	25	25	47.22	16.772	0.4193
14	40	25	15	35.00	11.449	0.1717
15	60	15	25	48.32	17.741	0.4435
16	50	20	20	44.45	16.378	0.3276

The extraction yield reached maximum of 50 % (run no.8) at 60 ℃ with extraction time and liquid to solid ratio of 15 min and 15, respectively. However, when the extraction time exceeds beyond 15 min (run no.6 and 13), the extraction yields did not increase with the increase of extraction time. This is mainly because of irradiation time

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affected the dielectric properties of solvents. As a result, extending the microwave exposure may cause degradation of targeted compounds due to overheating of the substances in the system [20]. Extraction yield does not solely depend on the extraction techniques but also influenced by amount of solvent used for extraction. The ratio of liquid-to-solid was optimized at solvent 15ml (run no.8). At this point, maximum amount of solvent penetrated into the solid by effective diffusion. Hence, the solute (of the blue dye) was effortlessly dissolved until approaching a concentration that restricted by the characteristics of the solid [21].

The value of *R*-squared for anthocyanin yield was R^2= *0.76633* (*>0.75*), indicating a good agreement between the experimental and predicted anthocyanin yield. Thus, the empirical model is adequate to explain most of the variability in the assay reading. The predicted second-order polynomial model for the anthocyanin yield (*Y*) fitted in the coded factors is shown in (4).

$$Y = -36.3623 + 1.9629X_1 + 2.4507X_2 - 0.8034X_3 - 0.0175X_1^2 - 0.0695X_2^2 + 0.0103X_3^2 + 0.0107X_1X_2 + 0.0063X_1X_3 + 0.0009X_2X \quad (4)$$

Where X_1, X_2 and X_3 correspond to the coded values of three independent variables of temperature (℃), time (min) and solid-liquid ratio (mg/mL), respectively.

Parity plot gives the correlation between the observed and predicted values and is shown in Fig. 2. Based on the parity plot, the regression model had low dispersion by explaining 76.63% of the total observed variation in the response. Thus, it can be concluded that all values are close to the regression line which serves as the reference line of the graph. Analysis of variance (ANOVA) was performed to evaluate the effect of variables and the potential interactions between them, at the same time to access the significance of the model. The adequacy of fitted model was checked by ANOVA using Fischer *F*-test as shown in Table III. The tabulated of *F*-values was based on (5).

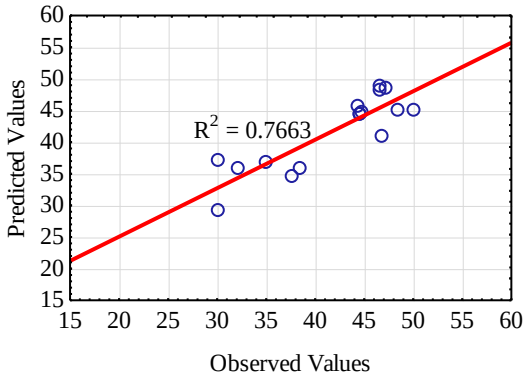

Figure 2. Parity plot

F-value (tabulated) = *F* (α, DoF_SSR, DoF_R) = *F* (0.05, 9, 6) = 4.10 (5)

TABLE III. ANOVA TABLE.

Factor	Sums of Squares (SS)	*F*-value (calculated)	*p*-value (calculated)
(1) Temperature (L)	430.6141	16.1873	0.0069
Temperature (Q)	32.0620	1.2052	0.3144
(2) Time (L)	18.0058	0.6769	0.4421
Time (Q)	31.4767	1.1832	0.3184
(3) Ratio (L)	1.0920	0.0410	0.8461
Ratio (Q)	0.6944	0.0261	0.7796
IL by 2L	2.2791	0.0857	0.7796
1L by 3L	0.8001	0.0301	0.8680
2L by 3L	0.0045	0.0002	0.9900

According to the *F*-Distribution table with α=0.05, *F*-value of *F* (0.05, 9, 6) is 4.10. Since *F*-value (calculated) of temperature (L) at 16.1873 higher than *F*-value (tabulated), it is statistically significant and the null hypothesis can be rejected. The effects of variables in pareto chart (*P*=95%) are shown in Fig. 3.

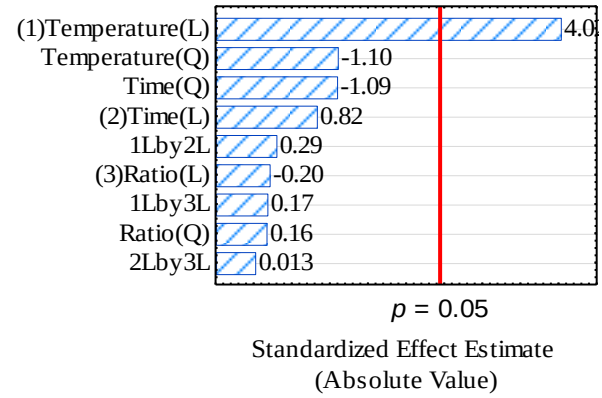

Figure 3. Pareto chart.

In regards to anthocyanin yield, when the *p*-value of X_1 was less than 0.05, it shows that temperature (L) was the significant factor that influenced the response. The temperature had shown a positive effect (*p*-value= 0.0069), denoting that an increase in temperature favoured the recovery of anthocyanin in the flower extract [22]. Thus, this analysis of the model obviously illustrates that the most influential factor was temperature. It is necessary to check the interactions between the model terms due to their significance of the model interactions. In addition, these interactions were depicted clearly by plotting two variables at a time on a contour (two-dimensional plot) as well as three dimensional surface plot [23].

Contour and three dimensional surface plots represent the interaction effect of temperature and liquid-to-solid ratio on anthocyanin yield as shown in Fig. 4 and 5, respectively. Based on Fig. 4 and 5, the extraction yield increased when both liquid-to-solid ratio and temperature increased. The increased of liquid in liquid-to-solid ratio from 10 to 20 mL/g was followed with an increased yield of total anthocyanin. This increase is probably due to the fact that more solvent can penetrate cells while more anthocyanins pigments can permeate into the solvent under the higher liquid-to-solid ratio conditions [24].

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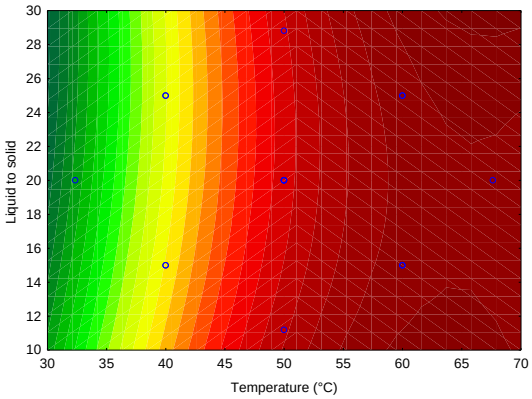

Figure 4. Contour plot of liquid-to-solid ratio versus temperature.

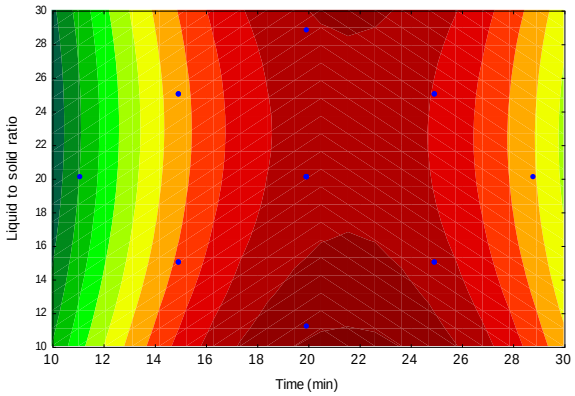

Figure 6. Contour plot of liquid-to-solid ratio versus time.

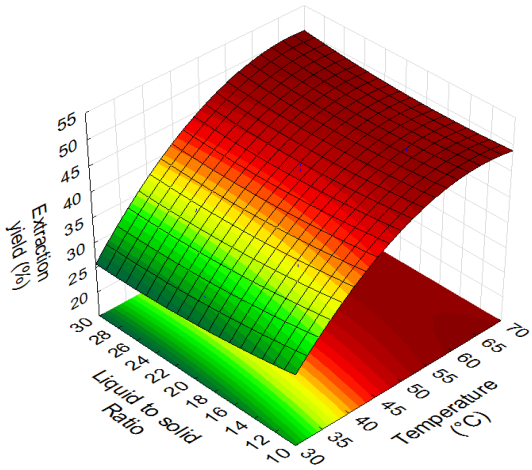

Figure 5. *3D*-plot of correlating yield, liquid-to-solid ratio and temperature.

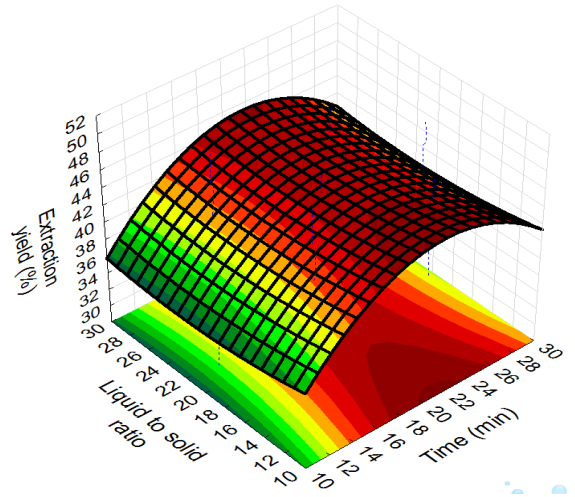

Figure 7. *3D*-plot of correlating yield, liquid-to-solid ratio and time.

A drop in total anthocyanin extraction yield was noticed when the further increase in liquid-to-solid ratio. This decrease is mainly because of the rising solvent proportion resulting lower solid weight ratio and decreased the density of extracted anthocyanin. Consequently, the total anthocyanin content was declined.

Contour and three dimensional surface plots represent the effect of interaction of liquid-to-solid ratio and time on anthocyanin yield as shown in Fig. 6 and 7, respectively. Both liquid-to-solid ratio and time had a quadratic effect on anthocyanin extraction. In scrutinizing the effect of solvent ratio and extraction time, the highest extraction yield was achieved regardless of solvent ratio but at intermediate time. The maximum total anthocyanin could be achieved when the liquid-to-solid ratio and extraction time were 20 mL/g and 20 min, respectively. The total anthocyanin yield increased with prolonged extraction time from 10 to 20 min. This observation was clear due to an extended irradiation time favours the extraction process of anthocyanin pigments [25]. However, the increase in time beyond optimum condition could decrease anthocyanin yield due to the effect of microwave radiation that possibly degraded the blue pigments.

Contour plot and three dimensional surface plots representing the interaction effect of time and temperature on anthocyanin yield are shown in Fig. 8 and 9, respectively. Time versus temperature was hyperbolic system of contours and optimized to give the highest total anthocyanin yield at extraction time 20 min and the temperature of 60 ℃. Throughout all the extraction time, rising the temperature reduced the total anthocyanin content linearly.

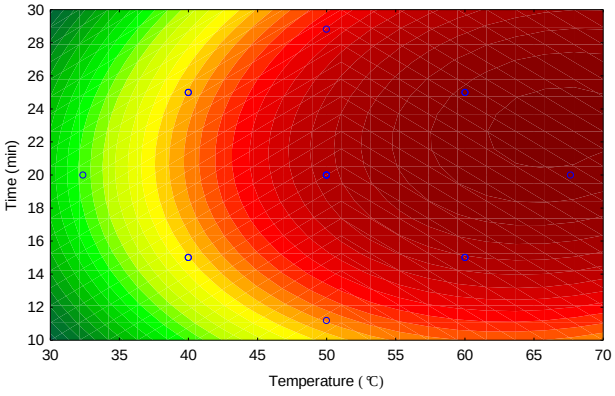

Figure 8. Contour plot of time versus temperature.

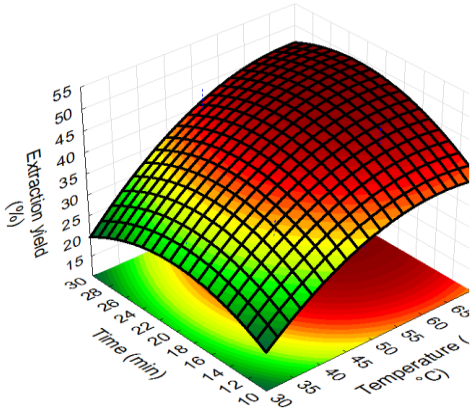

Figure 9. *3D*-plot of correlating yield, time and temperature.

This reduction was majorly due to the factors that monomeric anthocyanins in extreme temperatures could have been deteriorated and polymerized to produce brown or colourless pigments [26]. Thus, the stability of anthocyanin was reduced and diminished to the half-life and consequently reduce the extraction yield.

Thrice experiments for validation of model were performed in accordance with critical values to investigate the optimum conditions of the key operating parameters. The optimized critical value of variables and the validation data are shown in Table IV and V, respectively.

TABLE IV. THE OBSERVED VERSUS PREDICTED VALUES.

	Critical values; Variable: Extraction yield Solution: Saddle point Predicted value at solution: 49.36		
Factor	Observed minimum	Critical value	Observed maximum
Temperature	32.4	66.1	67.6
Time	11.2	22.8	28.8
Liquid-to-solid ratio	11.2	17.6	28.8

TABLE V. VALIDATION OF MODEL.

Factor	Value		
	Observed	Predicted	Error
Extraction Yield (%)	48.61	49.36	1.5

The predicted yield at the optimum conditions was obtained at 49.36%. Confirmatory experiments were conducted by giving the extraction yield at 48.61%. Consequently, the error difference between observed and predicted values was minimum at 1.5% and this small error percentage indicated that the model has high desirability [23]. Moreover, the model proved to be 99.48% accurate when compared with the predicted yield. Thus, the predicted and confirmation values verified that the optimal conditions were practical and had a good agreement with each other.

The extracted samples were analyzed using gas chromatography mass spectrometry (GC-MS) with a referral blank solution of the solvent. In the data of GC-MS, phytochemical screening of extracts displayed the presence of various bioactive compounds such as fatty acids, phenolic compounds, alcoholic compounds and organic acids [27]. However, other antioxidant compounds were identified between 40 to 46 min and the comparison between blank solution and samples was performed. Four major phytochemical components were identified namely nonacosane (1.33%), eicosane (3.61%), stigmasterol (6.37%), beta-Sitosterol (4.87%) were detected. The summary of identified components is shown in Table VI.

TABLE VI. SUMMARY OF IDENTIFIED COMPONENTS IN EXTRACTED FLOWER BY GC-MS.

Retention time (min)	Peak area (%)	Component	Molecular formula	Molecular weight (g/mol)	Ref.
41.042	1.33	Nonacosane	$C_{29}H_{60}$	408.80	[28]
43.585	3.61	Eicosane	$C_{20}H_{42}$	282.66	[29]
44.909	6.37	Stigmasterol	$C_{29}H_{48}O$	412.70	[30]
45.753	4.87	Beta-Sitosterol	$C_{29}H_{50}O$	414.72	[31]

IV. CONCLUSION

In this study, three process variables namely extraction temperature, liquid-to-solid ratio and extraction time were investigated and the extraction temperature remarkably affected the anthocyanin yield. When microwave-assisted extraction (MAE) was carried out at extraction temperature of 60 ℃, liquid-to solid ratio of 15:1 and an extraction time of 15 min, the total anthocyanin content (TAC) in the extract was 0.457 mg/g with the extraction yield of 49.97%. Based on the obtained results, MAE is an efficient method and can significantly maximize the extraction yield. Different compounds were analyzed from *Clitoria Ternatea* through GC-MS. Among the major phytochemicals detected in the samples were nonacosane (1.33%), eicosane (3.61%), stigmasterol (6.37%), as well as beta-Sitosterol (4.87%). The presence of these constituents in the plant justified and provided the scientific evidences to use the flower as natural dyes in food and beverage industry. Thus, these results suggested that pigments extracted from *Clitoria Ternatea* flower are naturally present in plant with high anthocyanin yield. In addition, since the flower can be propagated easily throughout the year so it can easily become a source of food colorant in market [32].

AUTHOR CONTRIBUTIONS

T. D. Munusamy conducted the experimental work for this research and wrote the paper; N.H. Hamidi and S.Z. Sulaiman analyzed the data; I. Izirwan revised and improved the paper; all authors had approved the final version.

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