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Factorial design for the effect of Ultrasound-Assisted Extraction (UAE) of *Labisia pumila sp.* in 25-L Mobile extractor

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ABSTRACT

Labisia pumila sp. is one of the herbs that have been widely utilized among Malay women in Malaysia. Traditionally, it is used as postpartum medication, health tonic and for body strength. In this study, a 2-level factorial experimental design was used to investigate the contribution of the main effect for the extraction of gallic acid in *Labisia pumila sp.* during ultrasound-assisted extraction (UAE) processes. The factors that have been preliminary screened were material to solvent ratio (1:20 and 1:40), temperature (50-80^{0}C), duty cycles (0-50%) and extraction time (4-8 hours) and validated statistically by analysis of variance (ANOVA). The extracts were analyzed by HPLC-DAD using a marker compound, gallic acid. This study was found that the duty cycle was the most influential factor and followed by the extraction time and temperature. All interactions between the studied factors were significant at 95% confidence level. Based on the results, the maximum yield of extraction was obtained with a temperature 80^{0}C, extraction time of 8 hours, the material to solvent of 1:20 and duty cycle 50%. At these conditions, extraction yield value determined as 105.731 ± 8.633 mg GAE/g dry weight. The experimental values under optimal condition were in a good consistent with the predicted values. Overall, the results obtained gives useful directions on how to improve recovery by proper selection of extraction condition.

INTRODUCTION

Since ancient times bioactive compounds in *Labisia pumila sp.* have been an object of interest to Man. The bioactive compounds are metabolites synthesized by plants for self-defense and other purposes Man used bioactive compounds for a various application (Munish *et al*., 2012). This *Labisia pumila sp.* has been used by generation of Malay women by boiling it in water. It is usually used as a tonic drink amongst women, and it is related with the phytoestrogen effects for women's health. Jamia (2000) claims that *Labisia pumila sp.* having a similar chemical structure to estrogen. Another essential point the tonic drink can induce and facilitate childbirth, as well as be a postpartum medication to help contract the birth channel and to regain body strength (Wan *et al*., 2007). Several studies have revealed that use of bioactive compound contributes to promoting optimal health and reduce the risk of critical disease (Denny *et al*., 2007). Adilah (2016) claims that gallic acid is one of important phytochemical in bioactive compound. Previous study reported that antioxidant activity of benzoic acid in gallic acid was higher than vitamin C and E against reactive oxygen species (Chua *et al*., 2012).

Nevertheless, the bioactive compound in plants is typically present at the low concentration (Stafford *et al*., 2002). Hence, an efficient and selective method for bioactive compound extraction is crucial part because solvent-based extraction of bioactive often suffers from low extraction yields, required long extraction times, and final product often contains traces of organic solvents, which decrease the product quality (Yang *et al*., 2011). Ultrasound-assisted extraction (UAE) of gallic acid from *Labisia pumila sp.* is a potential alternative

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method as compared to the conventional extraction solvent method. The application of sonication enhanced the efficiency of extraction even when the process was conducted for a shorter time. It is most likely due to the intensification of mass transport and ultrasonic is primarily attributed to the mechanical, cavitation and thermal forces disrupting cell walls (Wei and Yang, 2015). Equally important, UAE able to offer reduced solvent consumption, lower temperature and increasing the yield of the extraction in comparison with the conventional method (Pingret *et al*., 2013). Duty cycle

Therefore, this work is to evaluate the effect of temperature, materials to solvent ratio, variance duty cycle and extraction time for extraction of *Labisia pumila sp.* in mobile ultrasound-assisted extraction (UAE) system using factorial design. The usage of factorial designs has been to provide statistical analysis of the experimental results. Screening of the extraction factors were performed by using two full factorials which enables evaluation of independent variables (extraction factors) and any interactions with dependent variables (yield of gallic acid). It is also used to examine the relationship between response variables and a set quantitative experimental variables or factors 4^2

MATERIALS AND METHODS

A. Materials and method:

Acetonitrile and sulphuric acid were of analytical grade and obtained from Merck (Germany), Sigma-Aldrich (UK). Gallic acid (3,4,5-trihydroxybenzoic acid) was purchased from Sigma-Aldrich Co. (St. Louis, Mo, USA). *Labisia pumila sp.* leaves were purchased from Kedah, Malaysia. The samples were placed in air tight plastic bag and kept in the refrigerator (-20^{0}C) until ready to extraction and analysed.

B. Experimental set-up:

Chipping leaves were weighed at and 0.9 kg and transferred into a 25 L extraction vessel contains 19 L of extraction solvent. Water is used for extraction solvent. The extraction vessel made of stainless steel 316 and equipped with the ultrasonic homogenizer at 500W of constant power and 250 kHz of frequency. After setting the condition in the extractor chamber based on Table 1, the ultrasonic energy which is tangential air blower and heat energy is applied, the extraction process was started. The impacts of extraction condition including material/solvent ratio, temperature, extraction time, and duty cycle on the extraction efficiency of *Labisia pumila sp.* were evaluated. Duty cycle of 50% was equivalent to sonication for 5 s followed by a rest period (no sonication) of 10 s. Similarly, another investigation was carried out in the absence of ultrasound irradiation. After ultrasonic extraction, the aqueous extracts were filtered through Whatman filter No. 1 and the filtrates were analysed using HPLC. Each experiment was carried out in triplicates.

C. Factorial design methodology:

A total of 16 experiments were performed according to a full factorial design with four factors. The full factorial was employed for screening on four relevant reaction factors which are extraction time, percentage of duty cycles, ratio of materials to solvent and temperature. The experiments were carried out in randomized run order to determine characteristic response which is the yield percentage of gallic acid. The independent factors and results were taken as design as in Table 1. Table 1 shows the experimental matrix design and the results of the response factors studied. The experimental design and analysis of data were done using a commercial statistical package, by Design-Expert version 7.1 (State-Ease, Inc., Minneapolis, MN).

Table 1: Experimental matrix design and results obtained for each of the response of factors studied.

Run number	Input variables				Response
	T (^{0}C)	Material/solvent ratio	Duty Cycle (%)	Extraction time (hour)	% yield of gallic acid (mg GAE/g dry weight)
1	50	1:40	0	4	16.339
2	80	1:40	0	4	48.121
3	50	1:20	0	4	15.730
4	80	1:20	0	4	48.641
5	50	1:40	50	4	77.411
6	80	1:40	50	4	34.271
7	50	1:20	50	4	73.303
8	80	1:20	50	4	74.058
9	50	1:40	0	8	19.284
10	80	1:40	0	8	61.905
11	50	1:20	0	8	32.133
12	80	1:20	0	8	52.084
13	50	1:40	50	8	101.411
14	80	1:40	50	8	46.661
15	50	1:20	50	8	83.151
16	80	1:20	50	8	105.731

D. HPLC analysis:

The gallic acid was analyzed by using Agilent 1100 HPLC (USA) system equipped with diode array detector (DAD). The separation was performed using Phenomenex Prodigy 5μ (250 X 4.60mm) analytical column. The samples were diluted using Milli-Q ultrapure water (Millipore, USA) with dilution factor at 2. Some HPLC methods were available in the literature for the determination of gallic acid compound (Adilah *et al*. 2016). However, to find the most preferable method some modifications have been done to the existing method. The mobile phase was composed of solvent A (3% formic acid) and solvent B (100% acetonitrile). Isocratic solvents were carried out 90% A and 10% B. The flow rate kept constant at 1 mL per minute for a total run time of 10 minutes. The injection volume for all the samples was 20 μL and the detection wavelength of 278 nm was used for the detection of phenolic compound. Quantitative determinations were carried out using the calibration curve of the standard. Equation 1 is used to calculate yield of gallic acid.

$$\begin{matrix} Yield\ of \\ gallic\ acid \end{matrix} = \frac{concentration\ of\ gallic\ acid\ in\ sample \times volume\ of\ solvent \times dilution\ factor}{weight\ of\ raw\ material} \quad (1)$$

RESULT AND DISCUSSION

Factor affecting the extraction of *Labisia pumila sp:*

The main effects of factors studied in the system are demonstrated from Pareto charts in Figure 1. According to the Pareto chart, in which the bar lengths are proportional to the absolute value of the estimated effect with 95% confidence level, it was possible to verify that percent of duty cycle demonstrates the most significant effect on yield percentage of gallic acid. The effect of extraction time was almost quarter of percent of duty cycle effects while materials to solvent ratio was close to the percent of duty cycle effect. The interaction materials to solvent ratio and percent of duty cycle effects were very small in comparison with linear effect but it was also significant 95% confidence level.

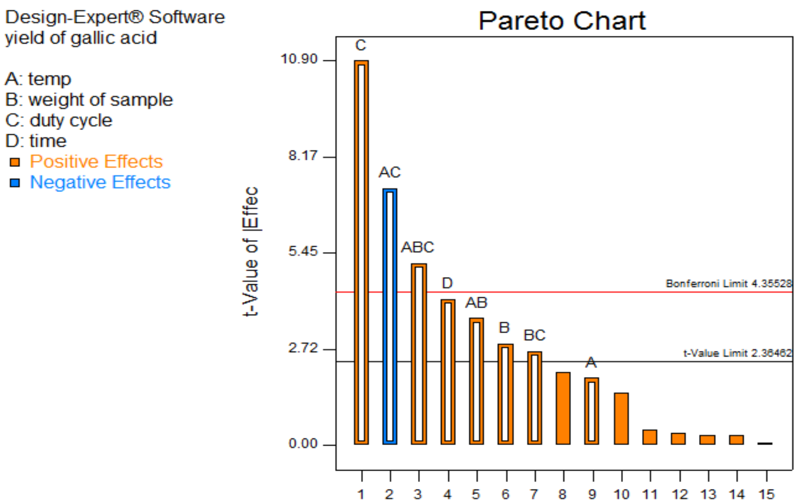

Fig. 1: Pareto chart for 2^4 factorial design.

In factorial analysis, contribution of the main factor gives an important effect in the optimization part later. One to two highest contributed factors will be selected from this factorial analysis. From Table 3 and Figure 1, factor C (duty cycles) gives the most contributing factor with 46.68 % to the UAE. Duty cycles indicate the availability of more gallic acid can be extract from *Labisia pumila sp.* This might because of the duty cycle enhance the mass transfer of gallic acid from the plant to the solvent. Moreover, the disruption of matrix increases the cell wall permeability, thereby enhancing the extraction of bioactive compounds towards extract (Munish *et al*., 2011). This finding is in agreement with Yolmeh *et al*., (2014) findings which showed yield of annatto seed extraction was increased as the duty cycles increased. Next, the second most contribution factor followed by 6.72 % of D (extraction time). Gallic acid production increased with at 8 h because large component concentration gradient between the solvent and raw material. At this point, may be explained by the compounds in the outer region of the plant particle being more easily extracted. However, long extraction times are not cost-effective due to their high-energy consumption. Moreover, in 2008 Ma *et al*., published a paper in which they described ultrasonic irradiation may damage the quality of heat-sensitive materials. After all the effect studied the highest production of phenolic compound was obtained at 105.731 ± 8.633 mg GAE/g dry weight where the extraction condition at 50% of duty cycle with temperature 80 ^{0}C, and 1:20 of materials to solvent for 8 h extraction time as shown in Table 1.

Table 3: Percentage of contribution of main factor on UAE

Factors	Contribution %
A	1.42
B	3.23
C	46.68
D	6.72

From Figure 1, all four factors (A, B, C and D) gave a positive effect (refer to orange bar chart) to UAE. It is suggested that the highest values will be used to favor the response. For example, an increase in the duty cycles (D) increases the yield of gallic acid. In contrast, the negative effect (blue bar chart) reveals that the use of the lower range value of factor will increase conversion to yield of gallic acid.

Analysis of variance (ANOVA) was performed by using Design Expert Software. Equation 3 shows the response surface quadratic model for gallic acid production which can be presented in terms of coded factors as in the following equation:

$$Y = 55.64 + (3.29x_1) + (4.96x_2) + (18.86x_3) + (7.16\ x_4) - (6.23x_1x_2) - (12.61x_1x_3) \\ - (4.60x_2x_3) + (8.92x_1x_2x_3 \quad (3)$$

Where Y was yield of gallic acid, x_1 was the temperature, x_2 was materials to solvent ratio, x_3 was percent of duty cycle, and x_4 was extraction time. The unknown x_1, x_2, x_3 and x_4 were referred to the main effects while x_1x_2, x_1x_3, x_2x_3 and $x_1x_2x_3$ were the interaction effects contributed in the UAE. Based on the quadratic model, coefficients of x_1 to x_4 are small compared to constant. This gives an indicator that the model equation is good with small error and can used for further analysis.

From the ANOVA of this experimental design as shown in Table 2, the model obtained was significant with P-value < 0.0001. The P-value was used as a tool to check the significance of each coefficient; according to Feng *et al*., (2010) the smaller the value of P, the more significant was the corresponding coefficient. Moreover, the F-value for a term compares a term's variance with the residual variance. The P-value <0.0001 means there was less than 0.01% chance that a model F-value could occur due to noise. Table 2 shows the model F-value of 30.93 implies the model is significant. P-values less than 0.05 indicate model terms are significant. For any term in the model, a large F-value and a small P-value indicated a more significant effect on the respective response variable (Ahmad *et al*., 2012)

Table 2: Test of significance for regression coefficient.

Source	Mean square	F-value	p-Value
Model	1482.13	30.93	<0.0001
x_1, Temperature	173.65	3.62	0.0987
x_2, Material/Solvent ratio	394.30	8.23	0.0240
x_3, Duty cycle	5691.19	118.76	<0.0001
x_4, time	819.19	17.09	0.0044
x_1x_2	621.05	12.96	0.0087
x_1x_3	2545.7	53.12	0.0002
x_2x_3	338.10	7.06	0.0326
$x_1x_2x_3$	1273.89	26.58	0.0013

The goodness of fit of the model was evaluated by the coefficient of determination (R^2), adjusted-R^2, and predicted-R^2. Coefficient of determination R^2 is the proportion of variation in the response attributed to the model. It is suggested R^2 should be close to 1 for a good fit model. The estimated model for percentage yield of gallic acid had satisfactory R^2 values of more than 90%, however a large value of R^2 does not always imply that the regression is good one. R^2 always increases with the addition of a new variable to the model, regardless of whether additional variables are statistically significant or not. Thus, it is preferred to use the adjusted-R^2 to evaluate the model adequacy since it is adjusted for the number of terms in the model. The adjusted-R^2 should be over 90% indicating a high degree of correlation between the observed and predicted values. For current finding the coefficient of determination (R^2) value obtained in this model was 0.9725 which is in good agreement with the adjusted R^2 value of 0.9410. The high R^2 value of 0.9725 indicates that the model was well adapted to the response.

Validation run:

The validation experiments were conducted based on one suggested best condition from Design Expert 7.0 in triplicate. The experiments were performed at 50 % of duty cycles, 1:40 materials to solvent, temperature at 50^{0}C and extraction time at 4 h and the results is presented in Table 4. The error from these validation runs in between 1.01 % to 2.13 % the model was found to be reliable and reproducible as the experimental values were in good agreement with the predicted values proposed by the model with an error less than 10%. Thus, it was proved to be an adequate model.

Table 4: Validation run for materials to solvent at 1:40, 50% of duty cycles, at 50^{0}C for 4 h.

Description	Yield of gallic acid (mg GAE / g dry weight)		
	Run 1	Run 2	Run 3
Predicted value	82.246	82.246	82.246
Experimental value	81.426±3.18	80.533±4.22	83.977±4.82
Error	1.01 %	2.13 %	2.06 %

Conclusion:

The use of UAE was found to have a significant effect on extraction efficiency of gallic acid obtained from *Labisia pumila sp.* extract. Based on the experimental results, it has been shown that different dependent responses favor different material to solvent ratio, temperature, extraction time and duty cycles. The results obtained from this study clearly indicate that the best condition for the UAE of *Labisia pumila sp. sp.* at 50% of duty cycles, 1:20 materials to solvent, temperature at 80^{0}C and 8 h of extraction time which produced 105.731 ± 8.633 mg GAE/g dry weight. As for duty cycle, operation in pulse mode may be useful and more favorable than continuous mode to reduce the electrical energy consumption of the whole extraction process. In this extraction of gallic acid, there are two factors that mostly contributed in UAE which are duty cycles and extraction time that will be used in the optimization part later. Hence, UAE is well known as an ‘environmentally friendly’ or ‘green’ technique and may have strong potential soon as an efficient process for the preparation of extract rich in natural antioxidant amidst growing environmental concerns.

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