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Optimization and Antioxidant Activity of Ultrasound-Assisted Extraction of *Piper betle* Oil

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Abstract

Piper betle belongs to the family Piperaceae and genus *Piper*. The essential oil in the leaves of the plant is heavily studied due to its biological properties, one of which being an antioxidant. Thus, this present study aims to optimise the essential oil extraction by hydrodistillation with ultrasonic-assisted extraction (UAE) as the sample pre-treatment. A Box-Behnken Design (BBD) of response surface methodology (RSM) was applied to study the effects of ultrasonic time (20-30 mins), extraction temperature (30-50°C) and solvent-to-solid ratio (20-40 mL/g) on the percentage yield of *P. betle* oil. The optimal conditions with the highest oil recovery (0.022% (w/w)) were found to be 25.6 mins, 44.5°C and 36.7 mL/g with R^2 value 0.7987. Antioxidant activity of *P. betle* oil were tested using free radical scavenging assay with 2,2-diphenyl-1-picrylhydrazyl (DPPH) as the reagent and ascorbic acid as the standard control. The IC_{50} value of the oil was determined to be 1.98 μg/mL. The values obtained in this study demonstrate the suitability of BBD model and DPPH assay to be used in optimisation of *P. betle* oil extraction and measurement of antioxidant activity, respectively.

Keywords : *Piper betle*, Optimisation, RSM, UAE, Antioxidant Activity

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1.0 INTRODUCTION

Piperaceae, or the pepper family, are pantropical plants and, as of 2017, consist of 3600 species in 13 genera [1]. These species are classified into two major genera, Piper and Peperomia where the genus Piper has approximately 2000 species. *Piper betle* belongs to the family Piperaceae and genus *Piper*. The betel vine is geographically native to India and Southeast Asia due to its cultivation in high relative humidity areas. The leaf of the plant has a physical appearance of shiny surface, cordate-shaped, aromatic scent and a taste that ranges from sweet to pungent. The leaf is also the leading component of the plant in *Piper betle* related studies [2].

Essential oil contains a complex mixture of volatile secondary metabolites, which consists of terpenoids and phenolic compounds as its main composition [3]. The presence of these constituents is the source of the distinct odour in aromatic plants like the *Piper betle*. The betel leaf contains around 0.08% to 0.2% of essential oil [2,4,5]. Not only the secondary metabolites present in the plant are functionally significant to the plant itself, but it is also useful to humans as antioxidants [6,7,8]. Ultrasound-assisted extraction (UAE) is a modern method commonly used as a sample pre-treatment. The penetration of solvent towards the sample is amplified from high speed collisions between the microscopic bubbles, causing them to burst rapidly at the plant cell. The cell wall is damaged thus releasing the essential oil from the plant into the medium. Consequently, extraction yield is high in a short span of extraction time [9].

The parameters that are commonly optimised for an essential oil extraction are extraction time [10,11], particle size [12,13], solid-to-solvent ratio [11,14}, sonication power [15], temperature [6] and ultrasonic time [15,17]. Extraction parameter is one of the factors affecting extraction yield. These parameters can be optimised using response surface methodology (RSM) where it applies a mathematical method to determine optimum values of experimental operating conditions for a target response based on factorial design. It builds a mathematical model to assess the relationship between variables [11,18]. One of the design types for RSM is Box-Behnken design (BBD), which has been applied in studies related to the effect of process variables and number of experiments [19]. Box-Behnken design (BBD) able to accurately determine the optimal value of experimental parameters, while simultaneously evaluating the interaction between variables.

Application of RSM for optimisation extraction of phytochemicals in plants were found to be effective [20], hence it is deemed suitable to be utilised in this study. This study aims to extract oil from fresh *Piper betle* leaves using UAE-hydrodistillation method. The extraction conditions of UAE would then be optimised using BBD with RSM. DPPH free radical scavenging assay in determination of antioxidant activity of *Piper betle* oil will also be conducted.

2.0 EXPERIMENTAL

2.1 Plant material

Fresh betel leaves were obtained from Sasta Florist in Skudai. The leaves were washed clean before being cut into small pieces ranging from 3 mm to 5 mm.

2.2 Sample pre-treatment of ultrasound-assisted extraction

Ultrasound-assisted extraction was done in an ultrasonic bath as stated by [30] with some modifications. Sample was subjected to ultrasonic waves with 100 W of power and 40 kHz of frequency under different variables (ultrasonic time (min), solvent-to-solid ratio (mL/g) and extraction temperature (°C). The value of power and frequency are based on the specifications of the ultrasonic model. The sample was weighed 100 g, 66.67 g and 50 g in a beaker containing solvent-to-solid ratios of 20, 30, and 40 mL/g. Distilled water was added into the beakers and the beakers were immersed in water bath. The sample were subjected to ultrasonic waves in the range of 20, 25, and 30 minutes with temperatures of 30, 40 and 50°C.

2.3 Extraction of essential oil by hydrodistillation

The sample went through hydrodistillation process using Clevenger apparatus after UAE to extract its essential oil with some modifications [11]. The sample was put in a 3 L round-bottomed flask containing 2 L of distilled water. The Clevenger apparatus consists of three main parts, connection with round-bottomed flask, condenser and separator. The separator separates the distilled water from the essential oil. The sample mixture was then heated using an electrothermal for 7 hours for maximum extraction. The essential oil was collected and separated using diethyl ether. The oil is then weighed, stored at 4°C and its percentage yield was calculated.

2.4 Experimental design by Box-Behnken design in response surface methodology

The optimization of the *P. betle* leaf oil was done using response surface methodology (RSM) according to [29] with some modifications. Design Expert-11 (Stat-Ease Inc., Minnesota, USA) was applied for data analysis in this study. The optimal combination of extraction parameters in this study were identified using three-level factorial Box Behnken Design (BBD). The ultrasonic time (X1), solvent-to-solid ratios (X2), and extraction temperature (X3) were the independent variables that has been optimized by BBD. The optimization of extraction was tested with different extraction time of 20, 25 and 30 minutes, solvent-to-solid ratios of 20, 30 and 40 mL/g and extraction temperature of 30, 40 and 50°C. The variables were optimized by RSM to obtain the highest amount of essential oil yield. The levels of each factor (high, +1, low, -1, and coded level, 0) that has been proposed by the software based on BBD approach, are shown in Table 2.1.

Table 2.1 The levels of each factor based on Box-Behnken design

Variables	Levels		
	+1	0	-1
X1: Ultrasonic time (min)	30	25	20
X2: Solvent-to-solid ratio (mL/g)	40	30	20
X3: Extraction temperature (°C)	50	40	30

2.5 DPPH free radical scavenging assay

The antioxidant activity of *P. betle* leaf oil was determined by free radical scavenging using 2,2-diphenyl-1-picrylhydrazyl (DPPH) with some modifications [15]. 100 µL of *P. betle* oil with concentration 1000 to 3.9063 ppm were reacted with 0.1 M methanolic DPPH solution. The reaction mixture was placed in the dark for 30 minutes to allow reaction to occur. Then, the absorbance was measured at 517 nm using spectrophotometer (Agilent, BioTek Epoch Microplate Reader California, USA). The test was carried out in duplicate.

3.0 RESULTS AND DISCUSSION

3.1 Optimization of ultrasound-assisted extraction (UAE) factors using response surface methodology (RSM)

The experimental conditions with the highest oil recovery (0.025% (w/w)) were found to be 30 mins, 40℃ and 40 mL/g. However, the optimal conditions demonstrated by RSM is 25.6 mins, 44.5℃ and 36.7 mL/g with an oil recovery of 0.022% (w/w). 25-30 minutes of ultrasonic time may be the favourable amount of time to spent for bubble cavitation to release essential oil from the plant into the medium. Lesser time than that may not be enough for the plant's cell wall to burst and promote mass transfer between the plant and solvent. High temperature causes the vapor pressure of solvent to increase but viscosity and surface tension decreases. This promotes the ultrasonic cavitation in UAE.

Table 3.1 Analysis of variance (ANOVA) for the quadratic polynomial model of percentage yield

Source	F-value	p-value	
Model	4.41	0.0149	Significant
X1	9.25	0.0124	
X2	14.26	0.0036	
X3	0.0990	0.7595	
Lack of fit	0.1018	0.9564	Not significant

*DF : Degree of freedom, X1 : Ultrasonic time, X2 : Solvent-to-solid ratio, X3 : Temperature

The low p-value (< 0.05) of the model as shown in Table 3.1 implies the significance and suitability of the model for the optimization study. Model terms can be indicated as significant when the p-values are less than 0.05 and insignificant when the values are greater than 0.1. In this study, both X1 (ultrasonic time) and X2 (solvent-to-solid ratio) variables' p-value are 0.0124 and 0.0036, respectively. This indicates that a change in those variables can have an influence on *P. betle* oil yield. However, p-value of X3 (0.7595) is greater than that of 0.1, hence it indicates that the variable is insignificant. The insignificance may be due to the reasons of low sample size, random variation or correlation with other variables. However, insignificance does not rule out the possibility of an effect, it just indicates that the data does not support the existence of one.

Table 3.1 shows the F-value for lack of fit is 0.1018 and p-value (0.9564) > 0.05. This shows that the lack of fit is not significant, indicating that the model fits well. This is also an indication that there is significant effect of parameters on output response. The observed and predicted values correlate with each other due to the value of the adjusted R^2 (0.6176) and R^2 (0.7987) being relatively in reasonable agreement with a difference of less than 0.2. Adequate precision measures signal-to-noise ratio with value more than 4 is considered desirable. The value of adequate precision is 6.87, hence it suggests an adequate signal. Overall, this regression model satisfactorily fit to the experimental data. The extraction is lower than previously reported yield for *P. betle* oil. This might be due to the differences in extraction conditions and sample preparation compared to previously reported literature.

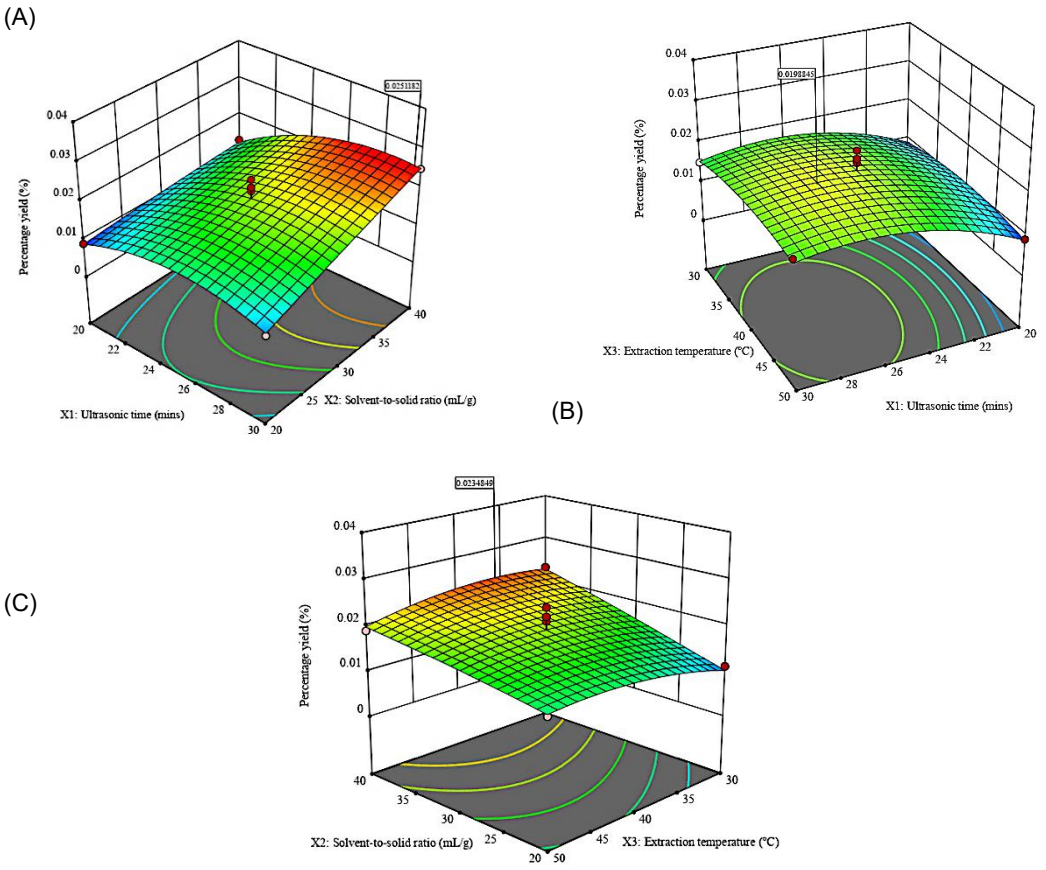


Figure 3.2 : 3D response surface plot on the effect of X1, X2 and X3 on percentage yield. (A) Effect of X1 and X2 on yield, (B) Effect of X1 and X3 on yield, (C) Effect of X2 and X3 on yield

Figure 3.2 shows 3D response surface plot that illustrates the interaction between any two independent variables (X1 = ultrasonic time (min), X2 = solvent-to-solid ratio (mL/g) and X3 = extraction temperature °C) towards percentage yield (%) by maintaining the third variable at zero level (midpoint). The warm-colored (red, orange and yellow) area indicates high yield while the cool-colored (blue and green) area is an indication of low yield. Figure 3.2 (A), (B) and (C) show the X1X2, X1X3 and X2X3 effects on yield, respectively. At constant extraction temperature of 40°C, it is found that the percentage yield was the highest at X1 = 30 mins and X2 = 40 mL/g while the lowest yield (0.009%) is at X1 = 20 mins and X2 = 20 mL/g with 0.009%. Figure 3.2 (A) shows percentage yield increases as both of ultrasonic time and solvent-to-solid ratio increases. It also indicates notable increase when ultrasonic time and solvent-to-solid ratio ranges from 24 to 40 mins and 33 to 40 mL/g, respectively. However, the interaction effect of (X1X2) was found to be significant as its p-value is more than 0.1 (0.1338).

Figure 3.2 (B) shows the interaction between ultrasonic time and extraction temperature on percentage yield of *P. betle* oil at constant solvent-to-solid ratio (30 mL/g). Based on the figure, X1X3 interaction has weak influence on the percentage yield, as shown by the less intense curve shape. As extraction temperature increases, the yield increases slightly but then decreases. An increase in ultrasonic time causes the yield to increase more significantly, but it started to decline at minute 28. This may be due to the increased probability of oil degradation by heat and oil evaporation into the surrounding.

At constant ultrasonic time of 25 minutes, the percentage yield was found to be the highest (0.023%) at 40 mL/g of solvent-to-solid ratio and 30°C of extraction temperature, as shown in Figure 3.2 (C). The figure shows a linear increase of percentage yield as X2 and X3 increases. Large ratio of solvent-to-solid can increase the rate of diffusion of essential oil because there is a reasonable concentration difference between the interior secretory cells and the solvent. However, the distance of diffusion could increase as the ratio gets larger.

Among all three variables, both ultrasonic time and solvent-to-solid ratio demonstrated p-values of 0.0124 and 0.0036, respectively. The values show that both variables are significant thus have much bigger impact on the percentage yield. The p-value for extraction temperature is 0.7595, indicating that it is non-significant, thus have lesser impact on percentage yield.

3.2 DPPH Free Radical Scavenging Activity

The *P. betle* oil from run 2 was selected for DPPH study because the yield extracted is the highest among the 20 runs. Figure 3.3 shows the relationship between DPPH scavenging percentage and concentration of *P. betle* oil. DPPH scavenging percentage increases as the concentration of oil increases. This could be due to the presence of more antioxidant constituents in higher concentration.

It was found that the IC_{50} value of *P. betle* oil is 1.98 μg/mL which is much lower than of ascorbic acid 26.02 μg/mL. The value is quite low when compared to an earlier study by [8], whose value is 3.6 μg/ml for *P. betle* of *Magahi* variant. But the IC_{50} value obtained in this study is closer to a later study by [7] whose value is 1.55 μg/ml for *P. betle* of *Maghai* variety. The low value of IC_{50} suggests the high antioxidant activity in *P. betle oil*.

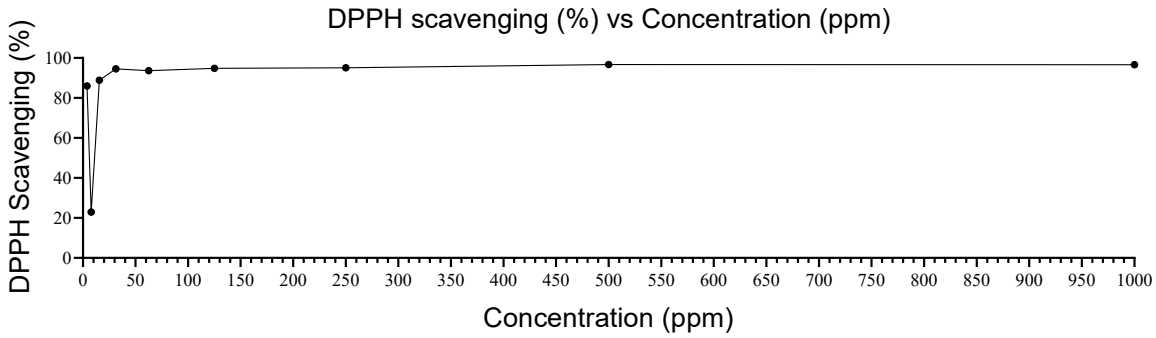


Figure 3.3 : Graph of DPPH scavenging (%) against concentration (ppm)

4.0 CONCLUSION

Response surface methodology (RSM) displayed its suitability to optimise the extraction parameters in this study thus demonstrated its role as a commonly used method in optimisation studies. The Box-Behnken Design (BBD) of RSM was used to find the optimal conditions for 3 different variables for 20 randomised experiments. The optimal conditions with the highest oil recovery (0.022% (w/w)) were found to be 25.6 mins, 44.5°C and 36.7 mL/g. The combination of ultrasonic time and solvent-to-solid ratio were determined as significant model terms as they showed p-values less than 0.05. The independent variables showed p-values less than 0.05 indicates the significance of the model terms. Thus, the variables give effect towards percentage yield of *P. betle* oil. A test of antioxidant activity was done using free radical scavenging assay with 2,2-diphenyl-1-picrylhydrazyl (DPPH) as the reagent. The IC_{50} value of the oil was determined to be 1.98 μg/mL, which is significantly lower than ascorbic acid's 26.02 μg/mL, indicating high antioxidant activity. The use of both RSM and UAE in this study indicates the efficiency and effectivity of the methods to determine the optimal conditions to extract essential oil.

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