Optimisation of Antioxidants Boiling Extraction from Pogostemon Cablin Benth.

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ABSTRACT

Background: Optimisation of antioxidants extractions from fresh samples of Pogostemon cablin Benth. from Kuantan, Malaysia by boiling method was conducted using response surface methodology. Objective: A 2^4 factorial design was applied to the independent variables boiling temperature (T) and boiling time duration (t), to find the area containing the maximum point of the total antioxidants activities (%), followed by composite design. The total antioxidants activities were determined using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay. Results: The results showed that the theoretical maximum antioxidant activity of the extract has the coordinates of temperature (T) at 116.18°C and boiling time duration (t) at 107.81 minutes, with the predicted antioxidant activity (%) at 68.6. Conclusion: Experiments conducted at these optimized levels of operational variables gave an average antioxidant activity-yield of 65.2%.

INTRODUCTION

For a practical application in industry, antioxidants from plants should first be extracted. The efficiency of the extraction process affects the antioxidant activity of the extract [1]. The Malay traditional practitioners have adopted the boiling method to extract useful components from plants/herbs in the production of jamu, a traditional medicine that is prepared from indigenous plants or herbs in the form of powder, pills, capsules, drinks and ointments. It is traditionally used to treat illness in the Malay Archipelago [2].

The extraction process aims to offer the maximum yield of substances (concentration of target compounds and antioxidant power of the extracts) at the highest quality. Pre-treatment of the sample (degreasing and size reduction), solvent/sample ratio, type of solvent, time and temperature of extraction are the variables that have been investigated up to now [3]. In order to minimize energy cost of the process, the time and temperature of extraction are important parameters to be optimized [4].

Many researchers accept the fact that an increase in the working temperature favours extraction, enhancing both the solubility of solute and the diffusion coefficient, but also that above a certain temperature phenolic compounds can be denatured [3]; [5]; [6]. Research by Spigno and De Faveri [3] showed that phenols yields at 60°C were higher than at 28°C, but an intermediate temperature of 45°C was chosen for the next trials in order to confirm if it would be likely to achieve the same result as for 60°C (or even better, in case a certain degree of thermal degradation occurred at 60°C), with reduction of energy costs. There are more contradictions in the data available for extraction time duration with some authors preferring quite short extraction times [5]; [6]; [7] while others chose quite long times [3]; [8]; [9]; [10]; [11].

The extraction efficiency is affected by multiple variables such as extraction temperature, extraction time, and solvent composition [12]. Processes are generally optimized using one-factor-at-a-time approaches. This traditional approach in fact is time-consuming [13]. Optimal conditions or interactions between variables cannot be predicted with this methodology. This limitation can be overcome using the method factorial experiments which are a specific form of Design of Experiment (DOE) [14]. DOE is a collection of statistical and mathematical techniques that have been successfully used in developing, improving and optimizing bioprocesses [15].
In this work, the variables that influence the extraction of antioxidants of Lamiaceae plants by boiling were investigated and the optimum levels of these variables were determined.

**MATERIALS AND METHODS**

**Plant Materials:**
Fresh samples of Pogostemon Cablin Benth. were collected from Kuantan, Malaysia. The plant was identified in the Biodiversity Unit, Institute of Bioscience, Universiti Putra Malaysia.

**Chemicals:**
1 Diphenyl-2-picrylhydrazyl (DPPH) was obtained from Sigma-Aldrich (Steinheim, Germany). Methanol (MeOH) was purchased from Merck (Darmstadt, Germany). The chemicals used were of analytical grade.

**Measurement of DPPH Scavenging Activity:**
Antioxidant activity assay are based on the measurement of the loss of DPPH colour at 517 nm after reaction with the test compound and the reaction is monitored by a UV-VIS spectrophotometer. The percentage of the remaining DPPH is calculated as % scavenging activity:

\[
\left(\frac{AA - AB}{AA}\right) \times 100\%
\]  

where AB is absorbance of DPPH\(^*\) solution in methanol, and AA is absorbance of a DPPH solution with a tested fraction solution.

In this assay, 0.4 ml of extract was diluted with 3.6 ml of distilled water. Then, from the dilution, 0.3 ml was placed in a test tube. 0.7 ml of methanol and then 2 ml of fresh methanolic solution of DPPH (0.004\%) were added. These solution mixture was mixed thoroughly and then kept in the dark for 30 min. The absorbance was measured at 517 nm using a UV-VIS spectrophotometer Model U-1800 (Hitachi, Tokyo, Japan).

**The Method of Factorial Experiment:**
There are actually many types of factorial experiments, but we will restrict ourselves to that involving 2 levels, ie the 2\(^n\) factorial experiments [16]. The method of factorial experiments has been designed to allow the effects of a number of experimental variables on the yield to be investigated simultaneously. It gives the “main effects” and the “interactive effects” of changing the experimental variables from a lower level to higher level. The main effect of an experimental variable is defined as the average of the effects of changing its value from the lower level to upper level among all the experiments. It is derived by assuming that the experimental variable is an independent variable and all the variations in its effect are due to experimental errors only. The interactive effects between two or more experimental variables are calculated on the assumption that the experimental variables are not independent but are in fact interacting between them.

The factorial experiments make use of a mathematical method known as the Yates’ Method (Yates [16]) to analyse the main effects and the interactive effects. In giving the main effects and the interactive effects the result of the analysis by Yates’ Method [17] also indicate whether the “yield response surface” in the area examined is curve or uncurved, and if it is uncurved, whether it is flat with the respect to the experimental variables or increasing or decreasing with respect to one or more experimental variables and if so, in which direction. The yield response surface itself is not actually a surface in the sense that a surface can only have a maximum of three dimensions whereas in this theoretical response surface the number of dimensions that can be considered is limitless. In this method, each of the experimental variables that is relevant to the yield is given 2 levels equidistant from the centre point, far enough from each other so that the effect of the difference in levels can be detected in the change in the yields. The levels of the experimental variables used in the 2\(^2\) factorial experiments, at \(\alpha = (-1)\) and \(\alpha = (+1)\), can be found in Table 1.

**Table 1:** Levels of Experimental Variables in the 2\(^2\) Factorial Experiments complimented with the Star Points making the Composite Design

<table>
<thead>
<tr>
<th>Experimental Variables</th>
<th>Levels of Experimental Variables</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>(a = -1.414) (a = -1) (a = 0) (a = +1) (a = +1.414)</td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td>39.5 50 75 100 111 168</td>
<td>(^\circ)C</td>
</tr>
</tbody>
</table>

Yates’ Method of Calculating the Main Effects and the Interactive Effects of the Experimental Variables in the Full Factorial Experiment:
To be able to use Yates’ Method [17], the experiments (consisting of various combinations of the experimental variables each in its upper or lower level) must first be arranged in the particular order as shown in experiments number 1 to 4 in Table 2, where \(a (-1)\) denotes the lower level and \(a (+1)\) denotes the higher level.
The (-1) and (+1) are actually representing particular levels ("a") where the change from (-1) to (+1) for an experimental variable was chosen on the basis that based on the then available information, this change should have given a large enough effect so as to be distinguishable from the experimental error and that the level of the experimental variable giving the maximum yield was most likely to be contained within this range. A 2^2 Experimental Design will comprise of 4 experiments as in experiments number 1 to 4 in Table 2.

<table>
<thead>
<tr>
<th>Experiment No</th>
<th>T (X₁)</th>
<th>t (X₂)</th>
<th>Antioxidant Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-1</td>
<td>-1</td>
<td>58.00</td>
</tr>
<tr>
<td>2</td>
<td>+1</td>
<td>-1</td>
<td>70.54</td>
</tr>
<tr>
<td>3</td>
<td>-1</td>
<td>+1</td>
<td>64.10</td>
</tr>
<tr>
<td>4</td>
<td>+1</td>
<td>+1</td>
<td>73.60</td>
</tr>
<tr>
<td>5</td>
<td>-1.414</td>
<td>0</td>
<td>62.81</td>
</tr>
<tr>
<td>6</td>
<td>+1.414</td>
<td>0</td>
<td>64.00</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>-1.414</td>
<td>65.72</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>+1.414</td>
<td>66.07</td>
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<td>0</td>
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<td>0</td>
<td>0</td>
<td>66.41</td>
</tr>
<tr>
<td>11</td>
<td>0</td>
<td>0</td>
<td>65.67</td>
</tr>
<tr>
<td>12</td>
<td>0</td>
<td>0</td>
<td>66.38</td>
</tr>
<tr>
<td>13</td>
<td>0</td>
<td>0</td>
<td>66.59</td>
</tr>
</tbody>
</table>

Average yield at Centre Point: 66.13
Sum of squared error at Centre Point: 66.13
Mean squared error (r^2) at Centre Point: 0.85
d.f. (n-1) of replicated Centre Point: 4

The combination of levels of variables as in experiments number 1 to 4 in Table 2 can be treated as a full 2^2 factorial using Yates’ Method [17] to calculate the main effects and the interactive effects using the following procedures:

Firstly, the combination of levels of variables are written down in the systematic order of the plan where the variables are introduced in turn and the introduction of any variable is followed by its combination with all the previous combinations of levels of variables (Table 3). Secondly, the yields are entered in the next column. To get the upper half of column 1 (i.e. number 1 to 2 of column 1 in Table 3) the yield are added in pairs. To get the lower half of column 1 (i.e. number 3 to 4 of column 1 in Table 3), the first number of each pair is subtracted from the second number. Thirdly, the same process is applied to column 1 to get column 2. The first number in column 3 is the grand total ("GT"). The succeeding factorial effects come out in the order in which the combination of the upper levels of variables were written down.

The Linear Approximate Equation of the Yield Response Surface and the Criterion for the Area Containing the Maximum Yield:

A linear regression between the yield of the antioxidant activity and the levels of the experimental variables in experiments 1 to 4 of Table 2 can be done on computer using the least square error method giving the form:

\[ y_N = a_0 + a_1x_i + a_2x_2 \]  

(2)

where \( y_N \) is the percent of antioxidant activity of the Nth experiment
\( x_i \) is the level of the ith experimental variable in the Nth experiment
\( a_0 \) is a constant
\( a_1 \) is the coefficient of the ith experimental variable

If the coefficients \( a_1 \) to \( a_2 \) are small compared to the constant \( a_0 \) and the fit of the equation is good (i.e. the errors are small), then the area investigated is a plateau which may contain the maximum. If the coefficients \( a_1 \) to \( a_2 \) are small compared to the constant \( a_0 \) but the fit of the equation is bad (i.e. the errors are large), the area is curved to both sides of the centre of the experiment and may contain the maximum yield. If the fit of the equation is good but the coefficients \( a_1 \) to \( a_2 \) are large compared to \( a_0 \), then the area investigated is steep and does not contain the maximum. If the fit of the equation is bad and the coefficients \( a_1 \) to \( a_2 \) are large compared to
a_0_0_ then the area does not contain the maximum. In these last two cases the Steepest Ascent Method [17] has to be used to find a new centre point and a new half-replicate 2^2 factorial experiment built around it.

The main effects interactive effects were then tested against error variance per experimental unit (“r”) which was derived by repeating the experiments at the centre point (α=0) as shown in Table 4.4, for significance at selected confidence level (95% and 99%) using the statistical F-test (Montgomery, 2001). The relevant values of the F-distribution are given in Table 4.5 and the results of the statistical F-test are given in Table 4.6.

**Method of Rotatable Composite Design:**

The method of Rotatable Composite Design [17] gives the complimentary experimental points which can be tested to enable the area containing the maximum (found as above) to be approximated by a quadratic equation and the levels of the variables at the maximum point evaluated using matrix algebra as suggested by [18]. The complete experimental plan [17] is called the “half-replicate 2^2 factorial plus star design and 5 points centre”. The star points are the four points at a distance α=(± 1.414) from the centre point. Here α is given the value of 1.414 to give the design the property of being rotatable, i.e. the standard error per unit experiment is the same for all points that are at the same distance from the centre of the region [17]. This is a property adopted because it was not known in advance how the response surface would orient itself with respect to the X-axes.

The form of the quadratic equation for 2-X variables is as follows:

\[
y_N = b_0 + \sum_{i=1}^{2} b_i x_{iN} + \sum_{i=1}^{2} b_{ii} x_{iN}^2 + \sum_{i=1}^{2} b_{ij} x_{iN} x_{jN}
\]  

(3)

where  

- \( b_0 \) is a constant  
- \( b_i \) is the coefficient of the ith experimental variable  
- \( b_{ii} \) is the coefficient of the square of the ith experimental variable  
- \( b_{ij} \) is the coefficient of the product of the ith experimental variable and the jth experimental variable.

The regression coefficients can be calculated on computer using SPSS Regression Analysis (Table 4).

To evaluate the levels of the experimental variables at the maximum point, the quadratic equation has to be written in full, which includes both the terms bij and bii, whereas in the calculation of the regression coefficients as above, the pair have been combined and written only once as bij since they contain the same variables in X. To eliminate any ambiguity each member of every pair of coefficients was replaced by their mean; (bij + bji)/2 [18], which is given by half the value of the coefficients as given in the regression above. The complete equation is therefore:

\[
y_N = b_0 + b_1 x_{1N} + b_2 x_{2N} + b_{11} x_{1N}^2 + \frac{b_{12}}{2} x_{1N} X_{2N} + \frac{b_{12}}{2} x_{2N} x_{1N} + b_{22} x_{2N}^2
\]  

(4)

This can be written in matrix notation as:
\[ Y_N = b_0 + B_i X + X^T B_{ii} X \]  \hspace{1cm} (5)

where \( B_i = \begin{bmatrix} b_1, b_2 \end{bmatrix} \)

\[ X = \begin{bmatrix} x_1 \\ x_2 \end{bmatrix} \]

and \( B_{ii} = \begin{bmatrix} b_{11} & b_{12} \\ b_{12} & b_{22} \end{bmatrix} \)

To get the coordinates of the maximum, the partial derivative of the response function was set to zero, the maximum was checked by using the second derivative and the simultaneous equations of the first derivatives were solved for the coordinates of the maximum point.

The partial derivatives of equation (4) can be expressed as follows:

\[ \frac{\partial y}{\partial x_i} = b_i + 2b_{ii} x_i + 2 \sum_{i \neq j \atop i < j} \left( \frac{b_{ij} x_j}{2} \right) \]  \hspace{1cm} (6)

which can be expressed in matrix notation as:

\[ B_i^T + 2B_{ii} X = 0 \]  \hspace{1cm} (7)

[18] [4] solved equation (7) for \( X_{\text{max}} \) giving the set of values of \( X_i \) at the point of maximum point as:

\[ X_{\text{max}} = -\left( \frac{1}{2} \right) B_{ii}^{-1} B_i^T \]  \hspace{1cm} (8)

The value of the theoretical maximum was calculated by introducing the values of \( X_i \) at the \( X_{\text{max}} \) into equation (5)

\[ y_{\text{max}} = b_0 + B_i X_{\text{max}} + X_{\text{max}}^T B_{ii} X_{\text{max}} \]  \hspace{1cm} (9)

Results:

Table 1 gives the levels of experimental variables in the \( 2^2 \) Factorial Experiments complimented with the Star Points making the Composite Design.

Table 2 gives the plan of the 22 Factorial Experiments with the Complimentary Star Points of the Composite Design and Five Replications of the Centre Point with the Experimental Results.

Table 3 gives the results of the calculation on main effects and interactive effects using Yates’ Method.

Table 4 gives the regression coefficients of the linear equation of the response surface of the 22 factorial experiments.

Table 5 gives the results of the F-test on the main effects and the interactive effects.

Table 6 gives the regression coefficients of the quadratic equation of response surface of the composite design.

Discussion:

Table 4 gives the regression coefficients of the linear equation for the response surface of the factorial experiment. The performance of this equation was evaluated by comparing the m.s. error of its prediction of the data of yield of experiments 1 to 4 in Table 2, with the m.s. error at the centre point, and it was found that the m.s. error of the prediction is significant at 99% on the F-test. All the coefficients of the linear equation are small compared to the constant. Despite the m.s. error of prediction being significant at 99%, since the
coefficients of the linear equation is small compared to the constant, the experiment were complemented with the extra data points (experiments 5 to 8 in Table 2) necessary to make it into a composite design [17]; [18]. The levels of the experimental variables used in these complementary points are as in Table 1, ie at points α = (±1.414).

Table 5 gives the results of the F-test on the main effects and the interactive effects of the experimental variables on the percentage of antioxidant activity. The main effect of T was significant at 99% confidence level, while the main effect of t was also significant at 99% confidence level.

The main effect of T has a (+ve) sign showing that increasing T from 50°C to 100°C has the main effect of increasing the antioxidant activity yield of the extract. Similarly for t its main effects has a (+ve) sign, thus increasing t from 60 minutes to 150 minutes has the main effect of increasing the antioxidant activity yield of the extract.

The response surface of the composite design was then approximated with a quadratic equation. Table 6 gives the regression constant and coefficients of the quadratic equation. This equation was then manipulated in the way explained in section The Method of Rotatable Composite Design to get the levels of the experimental variables at the theoretical maximum point.

The levels of the experimental variables X1 (Temperature) and X2 (Time) at the theoretical maximum point were calculated to be α = 1.6471 (equivalent to 116.8°C) and α = 0.0624 (equivalent to 107.81 minutes) respectively. The predicted yield of antioxidant activity is 68.6%. Experiments conducted in four replicates at these optimised levels of experimental variables give an average antioxidant activity -yield of 65.2%.

Conclusions:
The theoretical maximum point of yield of antioxidant activity of the extract obtained by boiling has the coordinates of temperature (T) at 116.18°C and boiling time duration (t) of 107.81 minutes, with the predicted antioxidant activity (%) at 68.6. Experiments conducted at these optimised levels of operational variables give an average antioxidant activity-yield of 65.2 %, ie close to the theoretical maximum.

References should be cited in the text as References: Bibliographic references in the text appear like [1, 2 ...], using square brace. References should be numbered consecutively in the text.

Authors are responsible for ensuring that the information in each reference is complete and accurate. All references must be numbered consecutively and citations of references in text should be identified using numbers in square brackets (e.g., “as discussed by Smith [9]”; “as discussed elsewhere [9, 10]”). All references should be cited within the text; otherwise, these references will be automatically removed.

No information writes in the paper without reference for authorization the information.

The list of references at the end of manuscript must be arranged consecutively and each reference in the list should appear in the following form:


Glossary of Terms

<table>
<thead>
<tr>
<th>No</th>
<th>Symbols</th>
<th>Meaning</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>X1</td>
<td>The experimental variable temperature</td>
<td>°C</td>
</tr>
<tr>
<td>2</td>
<td>x1</td>
<td>A value of X1</td>
<td>°C</td>
</tr>
<tr>
<td>3</td>
<td>_</td>
<td>The specific value of X1 at point P</td>
<td>°C</td>
</tr>
<tr>
<td>4</td>
<td>X2</td>
<td>The experimental variable time</td>
<td>%</td>
</tr>
<tr>
<td>5</td>
<td>x2</td>
<td>A value of X2</td>
<td>%</td>
</tr>
<tr>
<td>6</td>
<td>_</td>
<td>The specific value of X2 at point P</td>
<td>%</td>
</tr>
<tr>
<td>7</td>
<td>y</td>
<td>Yield of antioxidant activity</td>
<td>%</td>
</tr>
<tr>
<td>8</td>
<td>ℓ</td>
<td>Coefficient of the partial differential</td>
<td>No units</td>
</tr>
<tr>
<td>9</td>
<td>a0</td>
<td>Intercept of the linear regression equation</td>
<td>%</td>
</tr>
<tr>
<td>10</td>
<td>a1</td>
<td>Coefficient of the variables in the linear regression equation</td>
<td>%</td>
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</table>
ACKNOWLEDGEMENTS

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