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Extraction Optimization of Total Phenolics from Thai Basil (Ocimum Basilicum Var. Thyrsiflora) Leaves and Bioactivities of the Extract

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Extraction Optimization of total phenolics from Thai basil (*Ocimum basilicum* var. *thyrsiflora*) leaves and bioactivities of the extract

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Abstract. *Ocimum basilicum* var. *thyrsiflora* leaves which contains numerous bioactive compounds has been traditionally used as medicinal herbs. Aqueous methanol extract from basil have been found to have blood sugar lowering capacity and can be used to treat patients with diabetes. In this study, different conditions were investigated to extract total phenolic content (TPC) from Thai basil leaves. Response surface methodology using Box-Behnken design was employed to optimize and determine the effects of three independent variables of extraction process, namely sample powder/solvent ratio (1/40 – 1/200 g/mL), extraction time (10 - 60 min) and temperature (45 - 65°C) on the total phenolic content. The results indicated that the selected extraction variables have significant effects on the total phenolic content. The optimal extraction condition was determined as following: sample powder/solvent ratio of 1/139 g/mL, time of 39.4 min and temperature of 51.2°C. At this condition, the total phenolic and total flavonoid contents of the extract were 41.76 ± 0.21 mg GAE/g dry weight and 10.95 ± 0.3 mg RE/g dry weight, respectively. Its antioxidant capacity as 2,2-diphenyl-picrylhyrazyl (DPPH) scavenging capacity was 39.06 ± 1.1%, while its inhibitory capacities against alpha-amylase and amyloglucosidase were 47.80 ± 0.93% and 24.86 ± 1.21%, respectively. As the result, the extract of Thai basil leaves exhibited fairly high antioxidant capacity and enzymatic inhibition capacities as compared to other medicinal plants. Thus, the extract of Thai basil leaves may be used as a source of bioactive compounds for health benefits.

Keywords: *Ocimum basilicum var. thyrsiflora*, Box-Behnken design, alpha-amylase, amyloglucosidase.

1.Introduction

Food of plant origin has many antioxidants and bioactive compounds such as phenolics, vitamin, trace element. Therefore, higher intake of antioxidant-rich product can help people reduce oxidative stress in human body. There is numerous research that diet rich in food antioxidants especially from plants, herbs, spices and beverages shows protective effect on human health and reduce serious diseases [1]. Basil (*Ocimum basilicum* L.) is popular aromatic herbs, growing in many regions in the world, largest member of Lamiaceae family. Thai basil is a type basil native to Southeast Asia. It grows up to 45cm and has shiny green, slightly serrated, narrow leaves with a sweet. It is widely used in cooking as well as in traditional medicine in many countries such as India, Africa and southern Asia. Regarding pharmacological studies,

various *O. basilicum* extracts have antibacterial, antifungal, antioxidant activity. And, they also to treat diabetes through a mechanism based on their alpha-amylase and amyloglucosidase inhibiting activity [2]. Phenolic compounds are the major class of basil secondary metabolities that are contributed to antioxidant and anti-inflammatory activity of its extracts [3]. The antioxidant activity of phenolic compound could come from the ability to donate hydrogen or electron and delocalize the unpaired electron within the aromatic structure. In similar, basil contains high levels of phenolics compounds such as phenolic acids, flavonoid, caffeic acid, rosmarinic acid, tannin. Soxhlet and maceration methods are used to extract of antioxidants from solid plant materials [4]. Process optimization is usually required for the purpose of compete extraction of the compounds of interest and without undesirable their chemical modification. However, one-variable-at-a-time (OVAT) method, one kind of optimization, contains a lot of drawbacks because the interactive effects between the variables studied are ignored and it also requires the numerous number of experiments [5]. Response surface methodology (RSM) optimization has hence been widely used in order to overcome these problems [6]. In this methodology, the relationship between different factors and the levels of the effective factors can be represented by a surface in three dimensions that is called the response surface. RSM has been frequently used in the optimization of food manufacture processes such as annatto seeds [7, 8], olive [9], date seed [10]. Among many classes in RSM designs, the Box-Behnken design (BBD) has been used more popular because of its simple structure and high efficiency [11]. Besides antioxidant capacities, other functions of bioactive compounds are also of interest such as their effects on diabetes, which are evaluated through their inhibition of two enzymes α-amylase (present in pancreases and salvia) and α-glucosidase (present in small intestine) because which they have important roles in digestive system regarding to the hydrolysis of starch into sugar. According to [12], flavonoids and polyphenols in some plants can be potential in anti-diabetes such as herbal plants [13], Nepalese herb Pakhanbhed [12] and basil [3]. Therefore, to the best of our knowledge, there is no report on optimization of antioxidants extraction from basil using RSM technique and to get maximum total phenolic content. Moreover, the antioxidant and inhibitory capacity of the extract against starch-hydrolyzing enzymes of the extract was also investigated.

2. Materials and methods

2.1. Plant materials and chemicals

Thai raw basil was cultured in Long An city in Vietnam and harvested after 3 months drilling. In addition, the basil would be blanched in boiling water in 10 s. After that, the leaves were separated from stems, arranged on trays and put in a cabinet dryer and dried in 50°C on the same day. The chemicals used for the analyses of total phenolic content include 75% methanol, Folin-Ciocalteu reagent, sodium carbonate, gallic acid, rutin, DPPH, p-nitrophenyl-alpha-D-glucopyranoside and and alpha-amylase from *Aspergillus oryzae* (~30 U/mg) and amyloglucosidase from Asperigillus niger (≥300 U/mL) in this study was purchased from Merk or Sigma-Aldrich.

2.2 Methods

2.2.1 Extraction of phenolic compounds

The dried leaves (1g) was accurately weighted and mixed with 75% methanol. By following the Box-Behnken experimental design with the independent variables and the dependent response of the yield, namely total phenolic content (TPC). After shaking in the water bath for a certain temperature, time and ratio of solid to liquid. The mixture was then centrifuged at 5000 rpm for 5 min to collect the supernatant. The residues were re-extracted for two more times to completely extract the phenolic compounds. All supernatants were then combined and adjusted to 60 mL and stored at 4°C in the fridge until analyses.

2.2.2 Total phenolic content (TPC) determination

The total phenolic content was determined using methods described by [9]. Firstly, 300 µL diluted extract was mixed with 300 µL Folin-Ciocalteu reagent. After 2 min, 2.4 mL of 5% sodium carbonate solution was added. Secondly, the mixture sample was subsequently incubated at 25°C for 1 h in the dark. The absorbance

was measured at 760 nm. The standard calibration was made using gallic acid with concentrations 0, 20, 40, 60, 80 and 100 μg/mL.

2.2.3 Determination of total flavonoid content

The total flavonoid content was determined using the aluminum chloride colorimetric based on the method of [14]. With some modification, 0.5 mL of the extracts were first mixed with 1.5 mL 95% ethanol, 0.1 mL of aluminum chloride 10%, 0.1 mL of potassium acetate and 2.8 mL of distilled water. Then, these tubes were incubated at ambient temperature for 30 min. Eventually, the absorbance of the reaction mixtures was measured by a spectrophotometer at 415 nm. The standard calibration was made by using rutin solution with the concentrations of 20, 40, 60, 80 and 100 μg/mL.

2.2.4 DPPH radical scavenging assay

The antioxidant activity was determined using DPPH radical scavenging assay which was described by [15] Firstly, 0.1 mL of the extracts was mixed 3.9 mL of DPPH. Then, the mixture was kept in dark at ambient temperature. After 30 minutes, the absorbance was measured by a spectrophotometer at 515 nm. Blank was made by the mixture of 3.9 mL of DPPH and 0.1 mL of methanol. After all, the scavenging of DPPH was calculated according to the following equation of [16]

$$\%\ DPPH\ scavenging = \left(1 - \frac{A_{sample}}{A_{control}}\right) x\ 100$$

2.2.5 Alpha-amylase inhibitory assay

Alpha-amylase inhibition was determined by following the method of [12]. The mixture was mixed by adding 0.5ml of the extracts, 0.5ml of 0.02M sodium phosphate buffer (pH = 6.9 with 0.006M sodium chloride) containing α-amylase solution. The solvent then was incubated at 25°C for 10 min. After incubating, 0,5mL of 1% starch solution in 0,02 M sodium phosphate buffer was added and incubated at 25°C for 10 min. The enzyme reaction was stopped by adding 1mL of dinitrosalicylic acid 96 mM and incubated in boiling water 5 min and cooled to room temperature. After that, the mixture was diluted with 10 mL distilled water. The absorbance was measured at 540 by spectrophotometer. In the control sample was used with 0.5 mL of buffer solution instead of enzyme activity.

The enzyme inhibition capacity is calculated by equation:

$$\%\ Inhibition = \frac{1 - A_{540\ extract}}{A_{540\ control}}\ x\ 100$$

where: $A_{540\ extract}$ is the absorbance at 540nm in presence of extract, $A_{540control}$ is the absorbance at 540nm of the blank.

2.2.6 Amyloglucosidase inhibition assay

Amyloglucosidase inhibition activity was determined by the method of [17]. Firstly, the enzyme inhibition assay mixture was made by mixing 0.05mL p-Nitrophenyl a- D- Glucoside (pNPG) (10mg in 2mL phosphate buffer) with 0.01 mL of the extract. The reaction mixture was made up to 2.8mL by phosphate buffer (pH=6.8). After that, 0,02mL of enzyme amyloglucosidase (2mg in 1mL of phosphate buffer, 5.7U/mg; Sigma Aldrich, USA) was added into the mixture and incubated at 37°C for 60 min. The enzyme was deactivated at 90°C for 10 min. The control sample was prepared by buffer instead of the extract. The absorbance was measured at 405 nm using spectrophometer. The enzyme inhibition capacity was determined by following equation:

$$\%\ Inhibition = \frac{1 - A_{405\ extract}}{A_{405\ control}}\ x\ 100$$

2.2.7 Experimental design

Total phenolic content (TPC) of blanched leaves were extracted with 75% methanol and optimized using Box-Behnken design with three variables in table 1. In table 1, all the experiments were generated in duplicate

Table 1. Symbols and coded levels of three variables chosen for BBD

Variables	Symbol	Coded levels		
		-1	0	+1
Power/solvent ratio (g/mL)	X_1	1/40	1/120	1/200
Time (min)	X_2	10	35	60
Temperature (°C)	X_3	45	55	65

*Parameter coded forms -1, 0, +1 are the minimum point, centre point and maximum point, respectively for the independent parameters power/solvent ratio, time and temperature.

2.2.8 Statistical analysis

All data were the means of triplicate measurements. The statistical analyses were carried out using the SPSS software (Statistical Package for Social Sciences) version 22.0 with a level of confidence of 95%. Using Design expert software (version 11, State-Ease Inl., USA) employed for fitting the mathematical modeling.

3. Results and discussions

3.1 Experiment of Box-Behnken Design (BBD)

The BBD experiment was conducted to evaluate the combined effects of independent variables on the extraction of total phenolic content (TPC). There were 17 runs of experiment, including 5 center points according to BBD. Three experimental variables including power/solvent ratio, temperature and time extraction. Their levels were selected from previous screening tests and shown in Table 2. The results of total phenolic content for 17 runs are indicated in Table 2. The experiments no. 2, 4, 12, 17 provided the highest TPC values of around 43.7 mg GAE/g dry weight.

Table 2. BBD matrix for three variables and the observed response

Experiment number	Ratio (g/mL)	Time (min)	Temperature (°C)	Response (TPC) (mg GAE/g dry weight)
1	40	35	45	28.04
2	120	35	55	43.67
3	120	60	65	35.15
4	120	35	55	43.72
5	120	10	65	25.68
6	200	10	55	31.69
7	40	35	65	25.12
8	200	60	55	33.23
9	200	35	45	38.35
10	120	10	45	34.53

11	40	60	55	30.41
12	120	35	55	43.91
13	200	35	65	29.41
14	120	60	45	37.23
15	120	35	55	36,66
16	40	60	55	30,41
17	120	35	55	43,8

* Average value of duplicate experiments

3.2 Regression analysis

Based on the BBD experimental design model, an empirical relationship between the input variables and obtained experimental results was expressed by a second order polynomial equation with interaction terms. The model obtained in form of coded factors is given below.

$$Y \text{ (mg GAE/g dry weight)} = 42.35 + 3.66X_1 + 3.03X_2 - 2.85X_3 - 8.24X_1^2 - 5.32X_2^2 - 3.88X_3^2$$

Where Y is the predicted total phenolic content, X_1, X_2 and X_3 are the coded terms of three independent test variables, ratio of liquid to solid, time and extraction temperature, respectively.

The data of experiments then were analyzed by ANOVA (table 3) and the significance of the model was evaluated (table 4). Table 3 shows that p-value of the model is less than 0.05 and F-value is 11.61, indicating the high significance of the model. Moreover, the p-value of lack of fit of is 0.9934 (>0.05) indicating the lack of fit was not significant relative to the pure error. This means that the model is adequate to describe the experimental data. The determination coefficient R^2 of 0.94 indicates that only 6% of the total variations cannot be explained by the model. The high value of adjusted R^2 (0.858) also confirms the good fitness of the model. Furthermore, a high degree of precision and reliability of the experimental data are also indicated by the low value of coefficient variance (C.V% = 6.93). In overall, the model is adequate to predict the experimental data in the range of studied variables.

Table 3. Analysis of variance (ANOVA) for Box-behnken model

Source	Sum of squares	Df	Mean Square	F-value	p-value
Model	605.89	9	67.32	11.61	0.0019
Residual	40.6	7	5.8		
Lack of fit	0.1076	2	0.053	0.0066	0.9934
Pure error	40.49	5	8.1		
Cor total	646.48	16			
R^2	0.94				
Adjusted R^2	0.858				
C.V%	6.93				

The p-value and F-value were also used as a tool to evaluate the significant effect of each factor and the interaction effect between factors (Table 4). Smaller p-value (< 0.05) and higher F-value indicate the higher significance of the corresponding factors. All three independent factors (X_1, X_2 and X_3) and their quadratic terms (X_1^2, X_2^2 and X_3^2) significantly influence the extracted phenolic content within a 95% confidence level. However, the interaction among these factors is not significant.

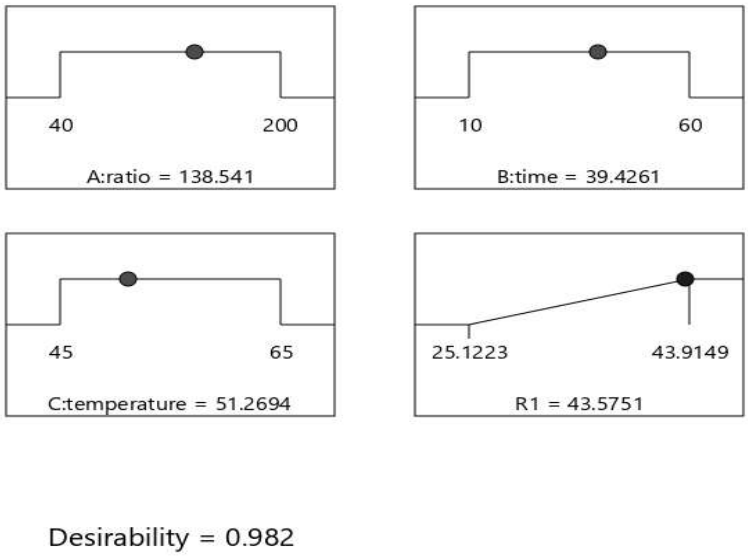
Table 4. Analysis of variance (ANOVA) for each factor

Source	Sum of Squares	df	Mean Square	F-value	p-value
A-Ratio	74.93	1	74.93	12.92	0,0088
B-Time	51.51	1	51.51	8.88	0.0205
C-Temp	64.98	1	64.98	11.21	0.0123
AB	10.96	1	10.96	1.89	0.2117
AC	9.06	1	9.06	1.56	0.2514
BC	11.47	1	11.47	1.98	0.2024
A^2	233.04	1	233.04	40.18	0.0004
B^2	97.30	1	97.30	16.78	0.0046
C^2	51.82	1	51.82	8.94	0.0202

3.3 Response surface plots

The model 3D response surface was the graphical representation of regression equation that show the relative effects of two variables. In addition, the shape of these contour plots also indicates that the interaction between the variables is significant or not. Circular shapes indicate negligible interactions while elliptical contours suggest significant interactions [18]. The 3D response surface and 2D contours generated by the model are shown in Figure 1, when two variables are depicted in each plot while the third variable is fixed at zero level. Figure 1 reveals that all three surface responses are convex in shape and their corresponding contours are elliptical, indicating that the ranges of three variables were selected properly

Figure 1 show the optimal condition to obtain the maximum TPC value, extracted from the surface responses using the Derringer's desired function methodology. This optimal condition includes the powder/solvent ratio of 1/138.5 (g/mL), extraction time of 39.4 min and temperature of 51.2°C. In order to validate the adequacy of the model equation, verification experiments were carried out under optimal conditions. However, considering the operability in actual production, the optimal conditions are modified as following: the ratio of powder/solvent ratio of 1/140 (g/ml), extraction time of 40 min and temperature 51°C. triplicate confirmatory experiments were performed and the experimental value was 41.75 ± 0.21 mg GAE/g DW (n = 3), obtained from the confirmatory experiments, demonstrated the validity of the RSM model, as there was no significant difference ($p>0.05$) between predicted value and experimental value hence indicating that the RSM model is satisfactory and accurate.



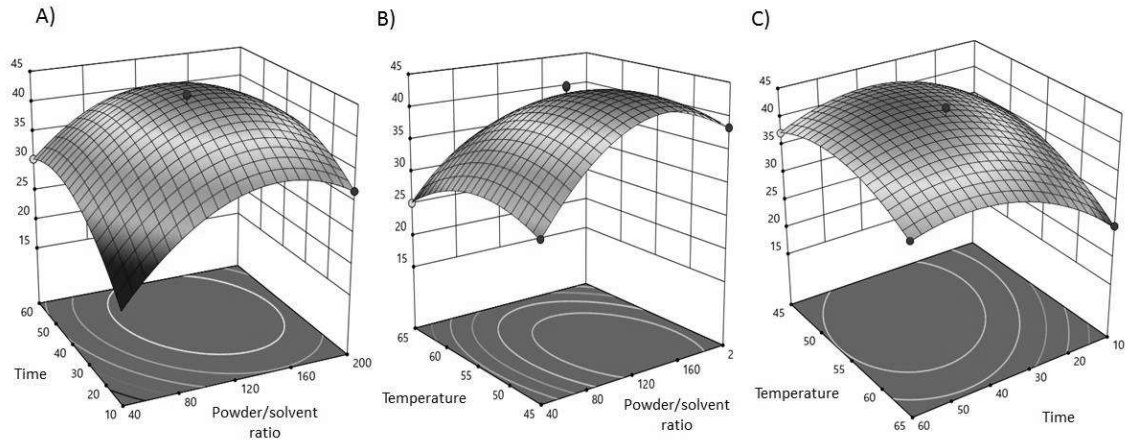


Figure 1. Desirability ramp for extraction process optimization

3.4 Antioxidant capacity, total flavonoid and enzyme inhibitory activity of Ocimum basilicum var. thyrsiflora's optimal extract

From Figure 2, inhibitory activities on both amylase and amyloglucosidase of the extract were high (47.80 ± 0.93% and 24.86 ± 1.21%, respectively), suggesting a desirable property against the starch – hydrolyzing enzymes. These high values were in accordance with its high TPC, TFC and DPPH values. As shown previously, the TPC content of the extract was 41.75 ± 0.21 mg GAE/g DW. On the other hand, the measured TFC value was 10.95 ± 0.32 mg RE/g (table 5). The scavenging activity tested by DPPH assay of the extract was nearly 40%, also. The inhibition of enzymes occurred as the phenolic compounds reacted with proteins to form insoluble complexes, resulting in the deformation of enzymes, thus preventing the binding ability with the substrates to release absorbable sugars [19]. In the research of Lam & Pham (2016), flavonoid content of bitter melon, especially the charantin content, could act to reduce the blood sugar, which was similar to the insulin function. El-Beshbishy & Bahashwan, (2012) revealed in their research on the *Ocimum basilicum* species that there was positive correlation between the DPPH scavenging ability and the inhibitory function of sucrase, pancreatic amylase and intestinal maltase; the inhibitory capability was substantial against maltase and sucrase, both of which were functionally equivalent to the α-glucosidase.

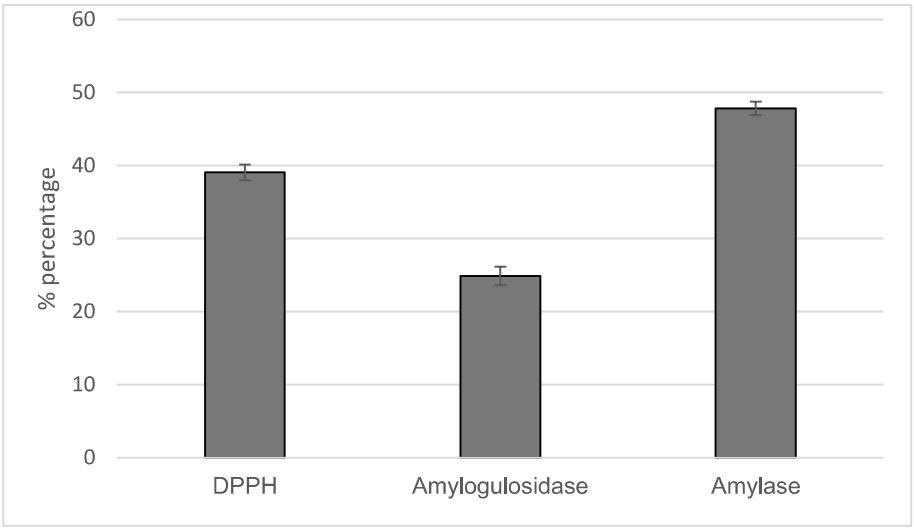


Figure 2. Antioxidant capacity, Amylogulosidase and amylase inhibitory activity of Thai basil leaves's extracts. Error bars represent the standard deviation.

Table 5: total phenolic content (TPC) and total flavonoid (TFC) of optimal extraction

Characteristics	**Value**
TPC	41.75 ± 0.21 mg GAE/g dry weight
TFC	10.95 ± 0.32 mg RE/g dry weight

4. Conclusions

In this study, we have successfully optimized antioxidants extraction from Thai basil using RSM technique and to get maximum total phenolic content. Results from RSM suggested that optimal conditions for this extraction including sample powder/solvent ratio of 1/139 (g/mL), time of 39.4 (min) and temperature of 51.2(°C). After verifying the predicted optimal yield, we determined that the actual yield is 41.75 ± 0.21 mg GAE/g dry weight. The antioxidant capacity and amylase inhibitory activity of the extract were high as compared to other medicinal plant. Therefore, the extract should be purified for obtaining the specific chemical compounds for diabetes disease.

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