Journal of Engineering Science and Technology
EURECA 2013 Special Issue August (2014) 79 - 88

OPTIMISATION OF EXTRACTION OF THYMOL FROM *PLECTRANTHUS AMBOINICUS* LEAVES USING RESPONSE SURFACE METHODOLOGY

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Abstract

Plectranthus amboinicus (PA) is one of the herbal plants that traditionally used to cure asthma, coughs and diseases affected by virus and bacteria. The objectives of this study were to extract essential oil from the leaves of this plant and optimise the extraction parameters using the Response Surface Methodology (RSM). The PA leaves were dried using a convection air drying method and followed by extraction using a Soxhlet method. Design Expert 6.0.8 was used to design the experiments based on the Box-Behnken Design with three parameters to control, viz. types of solvents, ratio of solid to solvent and extraction time. The types of solvents used were ethanol (95%), n-hexane (85%) and diethyl ether (99.5%). While for the ratio of solid to solvent were 1:30, 1:35 and 1:40. The extraction time was set in between 4 to 6 hours. The extracts obtained from different Soxhlet extraction parameters were concentrated to 5 ml using a rotary evaporator and analysed using a Gas Chromatography (GC) with a flame ionization detector. It was found that higher concentration of thymol was observed in the oil extracted using ethanol. The optimum extraction conditions were 6 hours with ratio of solid to solvent of 1:30.

Keywords: *Plectranthus amboinicus*, Essential oil, Soxhlet extraction, Gas chromate-graphy analysis, Response surface methodology (RSM)

1. Introduction

Herbal medicine has become part of the society that people sought after for medication purpose. It has been used in China for nearly 50 centuries. According to the report from Herbal Medical Database Ltd., the US market for herbal medicines has been estimated to be worth US$ 58 billion in 1999 with an annual growth rate of 25%.

Synthetic drugs do play an important role in enhancement of human living

Nomenclatures

DF	Degree of Freedom
GC	Gas Chromatography
PA	*Plectranthus amboinicus*
PRESS	Predicted Residual Sum of Squares
RSM	Response Surface Methodology

standards during the past century but recent years herbal medicine has earned its momentum once again as injection and consumption of synthetic medicine cannot cure the ailments; however, herbs can improve the body immune system to prevent the attack of chronic diseases [1] .

Treatment for chronic diseases with modern medicine or drugs might have various and severe adverse effect on patient's body. Therefore, people nowadays are looking for naturally originated agents with very little side effects to substitute chemical therapeutics. PA leaves are considered as one of the medicinal herbs. PA plant is a type of plant belongs to family of Lamiaceae which is known as borage in English [2]. PA leaves have been widely used traditionally for various purposes such as medical treatment for cough treatment, cephalagia, otalgia, anorexia, diarrhoea, cholera, chronic asthma malarial fever and cathartic [3]. The principal component of the oil is Thymol [4, 5].

Essential oil is the essence from a plant which is important for pharmaceuticals [6]. Thus, the concentration is a vital element in this study. An appropriate solvent is needed to maximise the amount of oils extracted. Due to this, the less polar solvent like ethanol, non-polar like hexane and diethyl ether were used to extract the targeted compound in the PA leaves, which is polar [7]. Throughout the research on essential oil from herbal leaves, the optimisation of the concentration of main active compounds from *Pletranthus amboinicus* using the Response Surface Methodology (RSM) has not been reported yet.

Therefore, the aims of this project were to analyse the concentration of the extracted essential oil from different extraction parameters using the Gas Chromatography (GC) and to optimise the extraction parameters using the RSM method.

2. Methodology

In this paper, the fresh plucked leaves were dried using a convective air drying method as by using this method, the air temperature can be controlled. This drying process was mainly for leaves moisture removal at a low temperature without deteriorate the extracted compound which is a heat sensitive compound. These dried leaves were later sent for the Soxhlet extraction. The solvents selected for the Soxhlet extraction were diethyl ether, hexane and ethanol. The main reason to select these solvents was based on their polarities. These solvents were categorized on the less polar and non-polar group and the targeted compound, thymol, is a polar compound. The extracts extracted from the leaves will be dried at boiling point of the solvents used in a rotary evaporator to 5 ml for every extracts. The extracts were stored at 4℃ and further analysis by using the GC.

2.1. RSM – *Box Behnken Design*

Design Expert version 6.0.8 was used to optimisation on concentration of the main active compounds from the essential oil extracted. The Box-Behnken Design (BBD) was selected as it demonstrated a more efficient design in optimisation [8]. There were 17 runs with different setting of parameters generated from the Design Expert (Version 6.0.8, Stat-Ease Inc., Minneapolis) statistical software.

2.2. Convective air drying

An oven (FAC-350, PROTECH, USA) was used to dry the leaves at low temperature, 50˚C for 72 hr in order for it to completely remove the moisture. The moisture loss of these leaves was found to be 94.6%.

2.3. Soxlet extraction

In this extraction method, solvents such as ethanol, diethyl ether and hexane were used. The main active compounds, thymol was found to be a polar [7]. The grinded dried leaves weighted about 6 g was placed in a cellulose thimble and inserted into the Soxhlet apparatus with 250 ml flasks. The 17 runs designed by the Design Expert 6.0.8 were performed based on the extraction time, ratio of solid to liquid and types of solvents designed for each run by the software. The solvent was added to the flask and heated and maintained in continuous reflux process. The extracts were concentrated to 5 ml by using a rotary evaporator (RV 10, IKA, USA) to separate the solvent from the essential oil. The essential oil was stored at 4˚C in universal bottle until being analysed.

2.4. Gas chromatography

GC (Clarus 500, Perkin Elmer, USA) analysis were carried out on Elite-5 MS capillary column (30 m × 0.25 mm × 0.25 µm of cross-linked Phenyl-Methyl Siloxane) equipped with flame ionization detector (FID). The initial column temperature was programmed at 60°C. It was raised at a rate of 10°C/min to 180°C for 12 minutes. The injector and detector temperatures were programmed at 260 and 280 °C respectively. Nitrogen was used as carrier gas with the flow rate at 2 ml/min.

2.5. Statistical analysis

Analysis of Variance (ANOVA) was used to test the significant terms in the model for each response and significant was judged by the F-statistic calculated from the data. Values of 'Prob > F' less than 0.05 indicate model terms are significant whereas values greater than 0.1 indicate the model terms are not significant.

3. Results and Discussion

In this study, the independent variables set were types of solvents (x1), extraction time (x2) and ratio of solid to solvents (x3). The response was the concentration of main active compounds, Thymol, extracted from different extraction conditions. The concentrations of thymol from PA leaves are shown in Table 1. The highest concentration of thymol was obtained using ethanol as the solvent (6 hours, ratio of 1:35). Thymol is a phytochemical that provides antioxidant activity and effective antimicrobial activity against food pathogens [9, 10]. The experimental conditions for diethyl ether did not show good results in terms of concentration of thymol could be due to its boiling point. It is predicted that its low boiling point, 34.6˚C could give higher reflux ratio and thus higher concentration of thymol could be obtained, but the prediction was incorrect. Based on the observation from the experiments, the evaporation rate of the diethyl ether is faster than its condensation rate and this has caused losses of diethyl ether vapor to the environment. So the extraction rate of diethyl ether is lesser than other solvents.

Table 1. Composition (%w/w) of Main Active Compounds of *Plectranthus amboinicus* Oils obtained Using Soxhlet Extraction.

Time (Hours)	Ratio of solid to liquid	Extraction Concentration (ppm)		
		Ethanol	Hexane	Diethyl Ether
		Thymol	Thymol	Thymol
4	1:30	1866.28	-	-
	1:35	1351.81	-	202.50
	1:40	1562.98	-	-
5	1:30	3339.40	-	376.57
	1:35	-	2209.91	-
	1:40	3616.28	-	210.57
6	1:30	-	2445.78	-
	1:35	3796.56	-	560.75
	1:40	-	2635.75	-

Extraction duration using ethanol as the solvent for 6 hr exhibited higher concentration of thymol compared to 4 hr. The Soxhlet extraction using ethanol yielded higher phenolic compounds concentration with the increase of the extraction time [11]. Thymol is very soluble in ethanol is because of thymol and ethanol form a strong hydrogen bonding [12]. Thymol, a phenol compound that has a chemical structure of an aromatic group attach to its functional group which is hydroxyl (OH) as shown in Fig. 1. While for the ethanol, it has a functional group of hydroxyl (OH) as well. So the oxygen from the ethanol compound will bond with the hydrogen from the phenol compound. Thus a hydrogen bonding is formed and this explains why thymol is very soluble in ethanol and why ethanol solvent can yield the highest concentration of thymol.

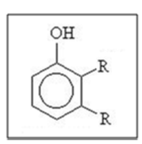

Fig. 1. Chemical Structure for Phenol Compound.

The model suggested by the Design Expert 6.0.8 was calculated using the statistical software. This result is shown in Table 2. In choosing the right model, the probability (Prob > F) needs to be examined, if the Prob > F falls below 0.05 then it should give the best model. In the fit summary result, Quadratic Model is suggested for this experiment as it has 0.0408 for its (Prob >F) and cubic model will not be considered even its Prob > F is 0.0002 as it has already aliased.

Table 2. Summary of Sequential Model Sum of Squares.

Source	Sum of squares	DF	Mean Square	F value	Prob >F	Selection of model
Mean	64116217.56	1	64116217.56			
Linear	16936330.02	3	5645443.34	22.65	< 0.0001	
2FI	1198235.06	3	399411.69	1.96	0.1846	
Quadratic	1371026.23	3	457008.74	4.77	0.0408	Suggested
Cubic	663550.57	3	221183.52	126.88	0.0002	Aliased
Residual	6972.89	4	1743.22			
Total	84292332.33	17	4958372.49			

DF: Degree of freedom

The "Lack of Fit Tests" compares the residual error to the "Pure Error" from replicated design points (RSM Tutorials Design Expert). Linear and 2FI models can be ruled out due to its Prob > F less than 0.0001. Since the cubic model is aliased, so it should not be considered. As identified from the Table 2 earlier, the quadratic model is likely to be the right model. This is further proved as it does not show significant lack of fit in Table 3.

Table 3. Lack of Fit Tests.

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	Selection of model
Linear	3232811.86	9	359201.32	206.06	< 0.0001	
2FI	2034576.80	6	339096.13	194.52	< 0.0001	
Quadratic	1371026.24	3	457008.75	4.77	0.0408	Suggested
Cubic	663550.57	3	221183.52	126.88	0.0002	Aliased
Pure Error	6972.89	4	1743.22			

The model summary statistics shows in Table 4 further listed out the other important information such as the standard deviation, R^2 value and PRESS value. A good model will exhibit low standard deviation, high R-squared value and low Predicted Residual Sum of Squares (PRESS) value. From this information, this has again proved that the quadratic model is the best model as it shows the characteristic of a good model by having low standard deviation, 309.50 compares to other models. Its R^2 value also is the highest, 0.97 by comparing to Linear and 2FI since Cubic is aliased. The closer the R^2 value to unity, the better the empirical model fits the actual data [13]. This suggested the predicted quadratic equation defined well the real behaviour of the system. In addition, the closer the adjusted R^2 value to the R^2 advocates for a high significant of the model [14]. It also has the lowest PRESS value at 10627704.24 comparing with Linear and 2FI models.

Table 4. Model Summary Statistics.

Source	Std. Dev.	R^2	Adjusted R^2	Predicted R^2	PRESS	Selection of model
Linear	499.21	0.8394	0.8024	0.6814	6.429E+006	
2FI	451.84	0.8988	0.8381	0.5528	9.023E+006	
Quadratic	309.50	0.9668	0.9240	0.4733	1.063E+007	Suggested
Cubic	41.75	0.9997	0.9986			Aliased

By applying multiple regression analysis on the experimental data, the concentration and the test variables are related by the following second-order polynomial in Eq. (1)

$$Y = 209.91 + 556.91A_1 + 1344.21\,B_2 - 0.31C_3 - 245.01A_1^2 - 487B_2^2 + 162.79C_3^2 + 521.63A_1B_2 + 123.32A_1C_3 + 110.72B_2C_3 \quad (1)$$

where A_1 is the extraction time, B_2 is the type of solvents, and C_3 is the ratio of solid to solvent.

Model terms that have Prob > F greater than 0.1 indicate the model terms are not significant and this could be improved by model reduction in order to achieve greater efficiency of the model selected. The Lack of Fit F-value of 126.88 implies the lack of fit is not significant as seen in Table 5. There is only a 0.02% chance that a Lack of Fit F-Value this large could occur due to noise. However, this could be improved by applying model reduction.

Table 5. Analysis of Variance (Partial Sum of Squares) for Quadratic Model.

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	Selection of model
Model	19505591.32	9	2167287.92	22.63	0.0002	significant
A	2481178.85	1	2481178.85	25.90	0.0014	
B	14455150.42	1	14455150.42	150.91	< 0.0001	
C	0.75	1	0.75	7.83E-06	0.9978	
A^2	252749.74	1	252749.74	2.64	0.1483	
B^2	998601.19	1	998601.19	10.43	0.0145	
C^2	111586.55	1	111586.55	1.16	0.3162	
AB	1088370.56	1	1088370.56	11.36	0.0119	
AC	60828.82	1	60828.82	0.64	0.4517	
BC	49035.67	1	49035.67	0.51	0.4975	
Residual	670523.46	7	95789.07			
Lack of Fit	663550.57	3	221183.52	126.88	0.0702	Not significant
Pure Error	6972.89	4	1743.22			
Cor Total	20176114.77	16				

DF: degree of freedom

Under the summary statistics from ANOVA as seen in Table 6, are shows that the Predicted R-Squared of 0.9032 is closed to the Adjusted R-Squared 0.9240 as expected. This may indicate a small block effect or a possible problem and data. Again, model reduction could improve the selected model. While for the Adequate Precision which measures the signal to noise ratio should above 4 so that it can be considered desirable. In this case, the Adequate Precision obtained was 16.018 and this indicated this model is significant for the extracting process [15].

From the contour plot seen in Fig. 2, it shows that at the extraction time of 6 hours and using type 3 solvents which is ethanol provided with the ratio at 2 which is 1:35 will give the highest predicted concentration of thymol from the essential oil at 3900.65 ppm. The Soxhlet extraction using ethanol yielded higher phenolic compounds concentration with the increase of the extraction time [11]. Essential oil extracted using hexane also shows lower concentration of thymol (2635.75 ppm) compare to ethanol (3796.56 ppm) but due to its toxicity of the hexane that would prevent its application in food products [16].

Table 6. Summary Statistics from ANOVA on Quadratic Model.

Std. Dev.	309.498086
Mean	1942.045882
C.V.	15.93670308
PRESS	10627704.24
R-Squared	0.966766473
Adj R-Squared	0.924037652
Pred R-Squared	0.903253183
Adeq Precision	16.01788468

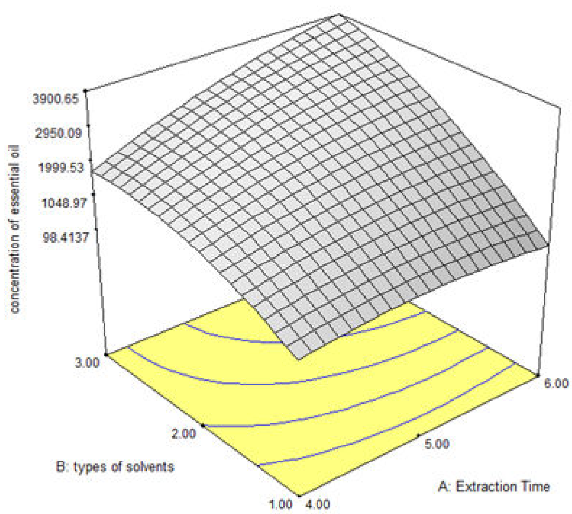

Fig. 2. 3D Contour Plot.

The standard error plot seen in Fig. 3 shows the variance associated with prediction changes over the design space. It is observed that Box-Behnken Design provides a good prediction as claimed by many researchers as there is a large area at the center point of the plot which shows lower standard error on the quadratic model selected. Moreover, the circular lines below the 3D contour plot also indicate the statistical property of rotability. Shaded region is noticed around the corner of the 3D contour plot, it means that these areas cannot be predicted and it serves as a warning for extrapolation. Overall, the quadratic model has lower standard error.

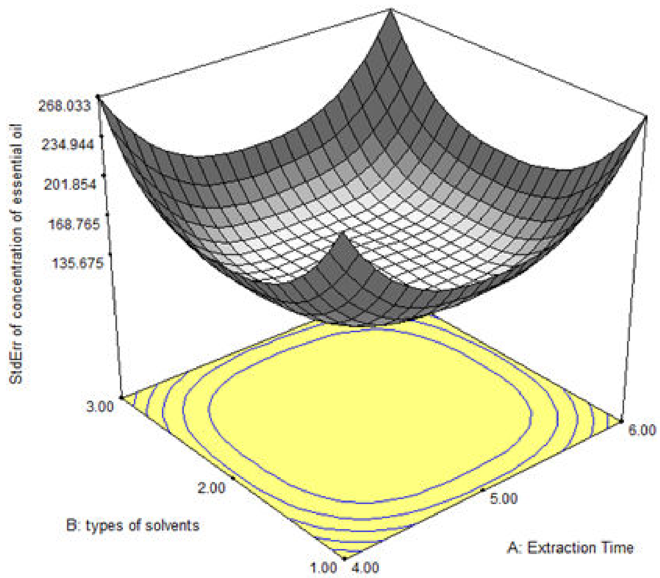

Fig. 3. 3D Plot for Standard Error.

The result obtained from the Table 1 could only show the best result. So it is further analysed to determine the optimise result. The optimisation result is shown in Table 7.

Table 7. Optimisation Result of Thymol Concentration Based on Optimised Numerical Setting.

Run	Extraction Time (hour)	Types of solvents	Ratio of solid to solvent	Concentration of thymol (ppm)	Selection of model
1	6	3	1	3804.4	Selected
2	6	3	3	3828.11	
3	6	3	1	3805.57	
4	6	3	3	3955.5	
5	6	3	1	3806.97	
6	6	3	2	3844.06	
7	6	3	2	4001.31	
8	6	3	1	3798.45	
9	6	3	3	3900.1	
10	6	3	2	3812.09	

It was found that the Run 1 was selected by the software as it has the optimum extraction parameters and gave 3804.40 ppm. Even though, Run 7 gave the highest concentration of thymol, 4001.31 ppm, but it was not selected by the software. This could be due to the insignificant increase of thymol concentration as the difference among them was 196.91ppm. By selecting Run 1, it can reduce the amount of solvent consumption by 37 ml or 21% and thus bring economic benefit to the project in term of low solvent usage and low waste produced. The high optimum extraction time in this experiment was found to be 6 hours [11]. The author found that ethanol was the solvent that able to extract higher concentration of thymol compared to hexane. This is in agreement with findings obtained by Kawase et al. [16] in extracting of *Origanum Vulgare L.* In addition, Nie et al. [17] claimed that the optimum ratio of solid to solvent was found to be 1: 30.

4. Conclusions

In this study, ethanol is the solvent that extracted highest amount of thymol at the ratio of 1:30 with 6 hours of extraction time meanwhile Diethyl ether is vice versa. Respond surface and the contour plots generated from the RSM used to determine the model for the experiment and the optimum zone within the experimental region. The proposed model was found to be a quadratic model. Based on the ANOVA analysis, the selected quadratic model gave low standard deviation, high R^2 and low PRESS value. The GC analysis was performed on the extracted essential oil and the main component of the leaves was found to be thymol. Data obtained from this study indicate application of RSM could optimise the concentration of thymol.

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