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Research Article

OPTIMIZATION OF EXTRACTION CONDITIONS FOR ANDROGRAPHOLIDE USING FRACTIONAL FACTORIAL DESIGN

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ABSTRACT

Andrographolide is a major bioctive compound found in king of bitter (*Andrographis paniculata*). In this study, the extraction method and its condition were investigated in order to get an extract with maximum amount of andrographolide by comparing three other extraction methods, i.e. maceration, soxhletation and ultrasonication and also determination for the optimum condition of the selected extraction method. The highest andrographolide amount was found by maceration, so this method was choosen for further optimization of extraction condition. The optimum condition based on the prediction amount from 27 factor combinations was obtained in 360 times of extraction time, 2g/100mL of sample to solvent ratio, and 3fold of extraction frequency with prediction of andrographolide amount was 3.50%. While by using prediction profile, the optimum condition was obtained in 360min of extraction time, 2 g/100mL of sample and solvent ratio, and 4 times of extraction frequency with the amount was 3.47-3.74%.

Key words: Andrographolide, king of bitter, *Andrographis paniculata*, fractional factorial design

INTRODUCTION

King of bitter (*Andrographis paniculata* Nees) is belongs to the family Acanthaceae and locally known as sambiloto. In Indonesia, sambiloto is widely used as ingredient in jamu (traditional Indonesian medicine). This plant usually used in jamu for the treatment of fever and itching in the skin, also as a tonic (BPOM, 2006). In addition, some studies show that king of bitter has a broad spectrum of biological activities such as anti-inflammatory (Shen *et al.*, 2002; Sheeja *et al.*, 2006), antioxidants (Sheeja *et al.*, 2006; Trivedi and Rawal 2001) antidiarrheal (Gupta *et al.*, 1993), antivirus (Wiart *et al.*, 2005), antimalarial (Dua *et al.*, 2004; Zein *et al.*, 2013), hepatoprotective (Rana and Avadhoot, 1991; Kapil *et al.*, 1993), anticancer (Matsuda *et al.*, 1994; Kumar *et al.*, 2004), antihyperglycemic (Soetarno *et al.*, 1999; Zhang and Tan, 2000; Reyes *et al.*, 2006), immunostimulant (Kumar *et al.*, 2004) and antibacterial (Singha *et al.*, 2003).

Major bioactive compound found in king of bitter are from diterpene lacton. Andrographolide (Figure 1) is known as one of the major bioactive compound present in king

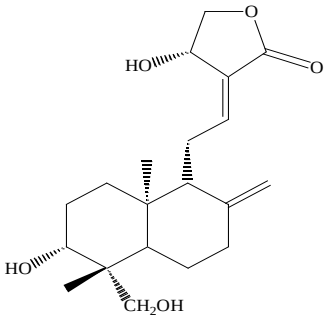

Figure 1.Chemical structure of andrographolide

of bitter (Jarukamjorn and Nemoto, 2008). Some biological activities such as anti-inflammatory (Shen *et al.*, 2000), antidiarrheal (Gupta *et al.*, 1993), anticancer (Sheeja *et al.*, 2007), inhibition of platelet aggregation (Amroyan *et al.*, 1999) and hepatoprotective (Singha *et al.*, 2007) are possesed. With these broad activities, it is necessary to have an extraction method with a maximum level of andrographolide in order to minimize the use of crude extract for a given dose based on the levels of andrographolide. Maceration, soxhletation and ultrasonication which have

been used extensively for andrographolide extraction. Each method has its own advantages and disadvantages. Therefore, we need to choose one from those methods and find optimum conditions that can extract andrographolide as much as possible.

This study aimed to find the best extraction method wth the optimum conditions for the extraction of andrographolide from king of bitter herbs using a randomized complete design (RCD) and fractional factorial design (FFD). Three extraction method namely maceration, soxhletation and ultrasonication were evaluated. RCD was used to choose an extraction method that give the highest yield of andrographolide. Further optimization of the selected extraction method was performd by evaluating variation the sample to solvent ratio, time and frequency of the extraction using RFF to reduce the number of experiment combinations.

MATERIAL AND METHODS

Apparatus

Maceration, soxhletation and ultrasonication extraction apparatus, rotary evaporator R-114 (Buchi, Switzerland), high performance liquid chromatography La Chrom Elite equipped with UV-Vis detector L-2420 (Hitachi, Japan).

Material and chemicals

King of bitter was obtained from Medicinal Plant Station of Biopharmaca Research Center, Bogor Agricultural University, Dramaga, Bogor. Dried samples were grounded to obtain 100 mesh particle size prior to be extracted. Andrographolide standard was purchased from Sigma-Aldrich (St. Louis, USA). Methanol p.a for extraction solvent and HPLC grade for the mobile phase were purchased from Merck (Darmstadt, Germany).

Maceration

Maceration method used in this study was referred to the method used by Akowuah *et al.* (Akowuah *et al.,* 2006). The experiment was performed in 250mL flask with 1g of powdered sample in 100mL methanol p.a. The extraction was carried out by soaking for 300min at room temperature and stirred constantly. The filtrate obtained was concentrated using a rotary evaporator. Extraction process was repeated three times.

Soxhletation

Soxhletation method reported by Wongkittipong *et al.* (Wongkittipong *et al.*, 2004) was used in this study. One gram powdered sample was soaked in 100mL of methanol p.a for 210min at 75°C and normal pressure. The filtrate obtained was concentrated using a rotary evaporator. Extraction process was performed in triplicate.

Ultrasonication

Ultrasonication method was referred to the method used by Yanfang *et al.* (Yanfang *et al.*, 2006). In a 250mL, 1g powdered sample with 100mL of methanol p.a. The mixture extracted for 30min using an ultrasonic bath at room temperature. The filtrate allowed to stand at room temperature for 30min before it was filtered. Extraction was repeated three times, after that the entire filtrate obtained was then concentrated using rotary evaporator. Triplicate extraction process was performed.

Determination of andrographolide

Andrographolide content was determined by HPLC according to the method developed by Akowuah *et al* 2006. Andrographolide was separated using a C18 column. Methanol-water (6:4 v/v) with pH 2.8 adjusted with phosphoric acid, was used as the mobile phase in isocratic mode. Temperature during elution was kept at 25°C, 20μL of injection volume and the flow rate of 1mL/min. Detection of andrographolide was performed at a wavelength of 210nm. An appropriate amount of standard stock solution was diluted with methanol to obtain three concentrations of the working standard solutions of the andrographolide for constructing the calibration curves. Andrographolide content was determined by using the calibration curve obtained previously.

Selection and optimization of extraction conditions

Selection of the extraction methods were evaluated using the RCD. F test is used to evaluate the effect of each factor to the

response under hypothesis null (H_0): all extraction methods have the same effect to the lconcentration of andrographolide. If $F_{observed} > F_{table}$, then H_0 is rejected, which means at least 1 extraction method affected the concentration of andrographolide. Further testing was performed in order to determine the most influential method in andrographolide content for choosing the selected extraction method. Selected extraction method obtained by RCD then optimized the conditions by evaluating the solvent ratio, extraction time, and the frequency of extraction using fractional factorial design 3^{k-1}.

Data analysis

Design experiment and data analysis was performed in SAS software version 9.1 (SAS Institute, North Carolina, USA)

RESULT AND DISCUSSIONS

Selection of extraction method

Three extraction methods were used namely maceration, soxhletation and ultrasonication to extract andrographolide. Differences from the three methods involved in the process of energy for extraction. Yield from maceration, soxhletation and ultrasonication methods are 33.3%, 30.8%, and 30.4% with andrographolide content are 2.83%, 2.23% and 1.86%, respectively. Extraction by maceration give the highest yield compared to the other extraction methods. This result indicated that the compounds in king of bitter are not resistant to heat or easy to decompose when exposed by ultrasonic waves. Other reason because andrographolide has a lactone ring making easyly to decompose if exposed to heating or ultrasonic waves.

RCD was used for selection an appropriate andrographolide extraction method which will be continued for further optimization of the extraction conditions with FFD. RCD is one factor experimental design with keep other factors in fixed condition. Application of one factor in the RCD usually used if the experimental conditions units are relatively homogeneous. It is generally used for the experiments conducted in the laboratory because the homogeneity of experimental units used can be guaranteed. Randomization was performed directly on the experimental units (Mattjik and Sumertajaya, 2002). From the results of data analysis using RCD showed coefficient of variance of the data was 4.71% showing that the experimental units used were relatively homogeneous. Futhermore, the three methods gave different mean value of andrographolide content at 95% significance level becausethe $F_{observed}$ (60.62) greater than F_{table} (5.14). To determine the effect of extraction method on andrographolide rendemend, Tukey test was performed another statistical test. This test was carried out by comparing all combination units on the error level of α with a relatively small number of combination units. The Tukey test results indicate that the three methods significantly different at the 95% significance level. Therefore, based on the results obtained, extraction using maceration method is a safe and simple technique to extract andrographolide in the optimum manner. In addition, we choosed the maceration method also due to the highly reactive and susceptible to rearrangement of the lactone ring.

Optimization of extraction conditions of maceration method

FFD was used for optimization of the conditions for maceration extraction of andrographolide. FFD is a derivative from the factorial design with reducing the number of combination trial. FFD will be obtained through a combination that only attributed for measuring the main effects while interaction from others are ignored. FFD will give the main characters needed to represent each factor. Each level of an attribute will be combined at each level of all factors with the amount or requency are proportional (Montgomerry, 2001). Extraction conditions such as the sample to solvent ratio, extraction time and the frequency were optimized using FFD $3k^{-1}$ which only performed one third of complete combinations (Table I). Early stage of optimization was determined the correlation between the yield of extract and andrographolide content. Based on statistical test for correlation, if p value $< \alpha$, there is a correlation between yield extracts with andrographolide content. Otherwise, there is no evidence to say any correlation between those two variables. As the p value = 0.595 was greater $\alpha = 0.05$, indicating

Table I. Fractional factorial design with 3^{k-1} and the experimental result

Obser-vation	Extrac-tion time (min)	Frequen-cy of extraction	Replicat e number	Weight of sample (g)	Weight of extract (g)	Sample to solvent ratio (g/100mL)	Androgra-pholide content (%)	Yield (%)
1	240	1	1	0.5001	0.1914	0.5	2.21	40.96
			2	0.5001	0.1869	0.5	2.51	40.00
2	300	5	1	0.5001	0.2221	0.5	2.38	47.53
			2	0.5003	0.2094	0.5	2.12	44.8
3	360	3	1	0.5003	0.2196	0.5	3.02	46.98
			2	0.5006	0.2172	0.5	2.91	46.44
4	240	5	1	1.0005	0.2990	1.0	2.46	31.99
			2	1.0013	0.3042	1.0	2.63	32.52
5	300	3	1	1.0007	0.3131	1.0	2.86	33.49
			2	1.0003	0.3419	1.0	2.95	36.58
6	360	1	1	1.0082	0.2360	1.0	2.57	25.05
			2	1.0024	0.2527	1.0	2.74	26.98
7	240	3	1	2.0007	0.5300	2.0	3.27	28.35
			2	2.0001	0.5977	2.0	3.25	31.98
8	300	1	1	2.0008	0.4068	2.0	2.61	21.76
			2	2.0027	0.3518	2.0	2.48	18.80
9	360	5	1	2.0002	0.5839	2.0	3.48	31.24
			2	2.0007	0.5763	2.0	3.54	30.83

Table II. Estimation of coefficient regression

Sources of variance	Estimation	Standard error	t	p-value
Intercept	1.7472	0.1818	9.61	<.0001*
Time	0.0021	0.0005	4.05	0.0016*
Frequency	0.0620	0.0160	3.86	0.0022*
Sample to solvent ratio	0.3890	0.0420	9.25	<.0001*
(Frequency-3)*(Frequency-3)	-0.0997	0.0139	-7.17	<.0001*
(Frequency-3)*(Sample to solvent ratio-1,16667)	0.1779	0.0259	6.84	<.0001*

*significant at $\alpha = 0.05$

no evidence any correlation between the yield of the extract and andrographolide.

The next step is to build a regression analysis using stepwise method for selecting main factors and interaction factors which most influence to the response (Table II). Time and the sample to solvent ratio were linier to andrographolide content, whereas frequency give quadratic effect on the level of andrographolide. There are significant interactions between frequency and sample to solvent ratio on the andrographolide content.

The goodness of a model could be determined from the coefficient determination (R^2). Ideally, a model is good if the value of R^2 near to 100%. In this study, the model has a R^2 about 94.91% which means that the model approximately the variance of andrographolide about 94.91% and only 5.19% explained by other factors.

Another approach for evaluation the goodness of the model is using the coefficient of variance. If the experiments conducted in the laboratory, the coefficient of variance for a good regression model must less than 10%. Coefficient of variance was calculated based on the ratio between the error divided by the mean response and then multiplied by 100%.

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*significant at $\alpha = 0.05$

Table III. Prediction on andrographolide content based on experiment which were not conducted

Sample to solvent ratio (g/100 mL)	Time (minute)	Frequency of extraction	Content* (%b/b)
0.5	240	3	2.65
0.5	240	5	2.14
0.5	300	1	2.50
0.5	300	3	2.78
0.5	360	1	2.63
0.5	360	5	2.40
1	240	1	2.38
1	240	3	2.85
1	300	1	2.51
1	300	5	2.64
1	360	3	3.11
1	360	5	2.78
2	240	1	2.42
2	240	5	3.26
2	300	3	3.37
2	300	5	3.39
2	360	1	2.68
2	360	3	3.50

*Obtained from regression model.

Error for this model was 0.11 and the mean content of andrographolide was 2.78%, so the coefficient of variance of the model was 4%.

Interaction effect between the sample to solvent ratio and frequency on andrographolide content is shown in figure 2. There are two sub-graphs in the figure, the left side in the first row is the effect of each frequency among the different levels of sample to solvent ratio. In this graph, the x-axis is sample to solvent ratio and the y-axis is andrographolide content mean. The effect of andrographolide content mean among the sample to solvent ratio is different between frequency 1 and frequency 5. The effect is flat when frequency is 1 and on the contrary, the effect of sample to solvent ratio in frequency 5 is linear.

The graph in the second row is different way to show the interaction effect between frequency and the sample to solvent ratio. The graph shows the effect of frequency to andrographolide content in various sample to solvent ratio. The quadratic effect of frequency to the response for solvent ratio 2 is different from the the quadratic effect for solvent ratio 0.5. When the solvent ratio equal to 2, maximum response was achieved when frequency is 4. Viceversa, when the solvent ratio equal to 0.5, maximum response was

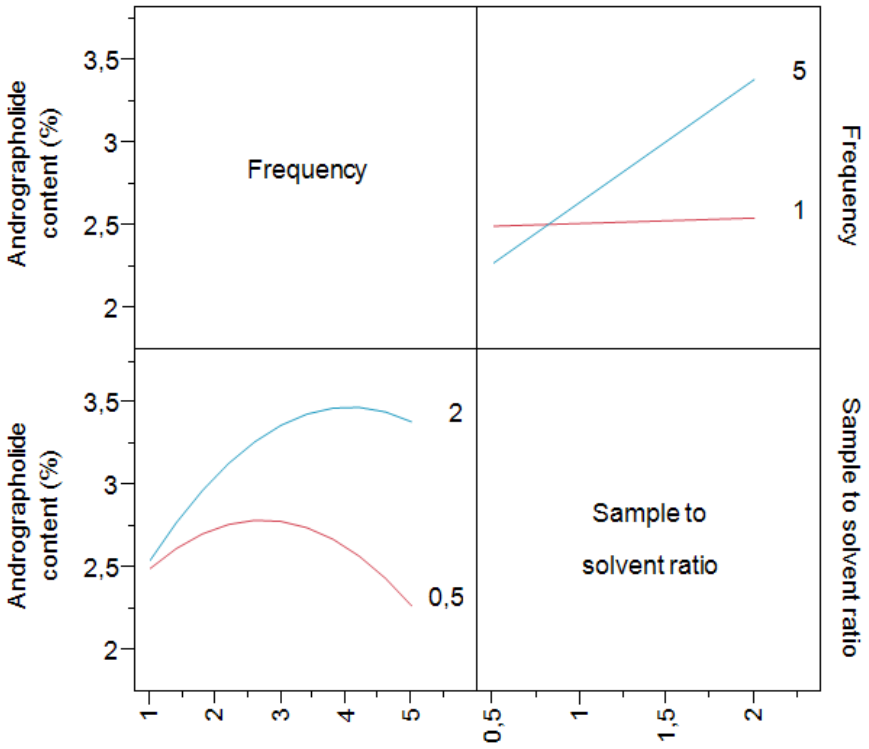

Figure 2. Plot of interactions effects of sample to solvent ratio and frequency to andrographolide content.

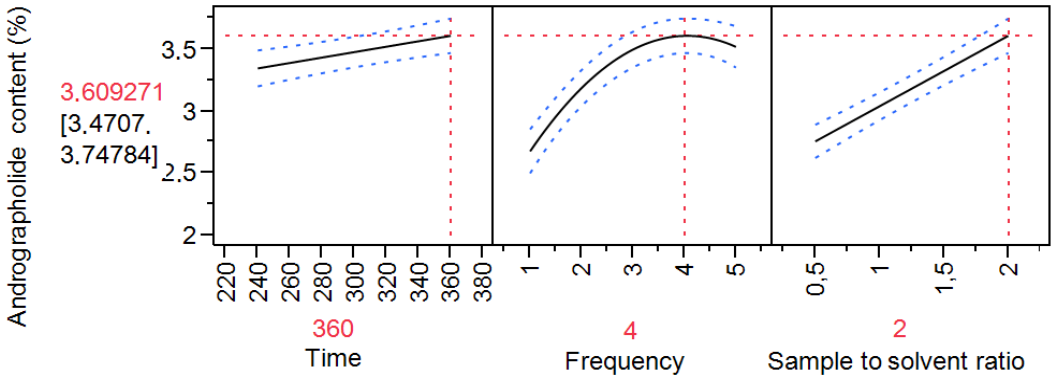

Figure 3. Prediction profile based on regression model

reached when frequency is 3 but was not as higher as when the solvent ratio = 2 was used.

Relationship between the sample to solvent ratio, time and frequency are shown by a prediction profiles (Figure 3). Prediction profiles showed the response prediction of a certain level of each factor based on the regression model was obtained. The figure contains three sub-figures which each sub-figure shows the relationship between each factor (x-axis) and the response (y-axis). The relationship between time and sample to solvent ratio to the response were linear, respectively. Meanwhile the relationship between frequency and the response was quadratic. The optimum condition was achieved with a sample to solvent ratio 2, extraction time 360min and frequency of 4 with

the andrographolide content ranging between 3.47-3.74%.

Furthermore, the response of other observation were predicted by regression model. Table 3 shows the prediction of of andrographolide from the combinations which are not conducted. The highest of andrographolide content (3.5%) was achieved with sample to solvent ratio 2, extraction time 360 minutes and frequency of extraction 3 times. Estimation under this conditions apparently give slightly lower andrographolide content when compared with the optimum conditions that estimated by prediction profiles.

Our finding showed a greater different andrographolide content compared to the result found by Mohan *et al.* (2013), maybe because we used a different type of extraction method. It is difficult to choose which one much better because we used sample from different location that will affect on the chemical composition of the sample. Nevertheless this finding could be used as an alternative method for extraction of andrographolide because of easy to perform and gave significant yield of andrographolide.

CONCLUSION

Androgapholide content extracted by maceration, soxhletation and ultrasonication are 2.83, 2.23 and 1.86%, respectively. Maceration method was selected to optimize the extraction conditions. Optimization conditions for maceration method showed sample to solvent ration and extraction time give a linear effect, whereas frequency give a quadratic effect on the andrographolide content. The optimum extraction conditions of andrographolide content based on 27 combinations, with the sample to solvent ratio 2g/100 mL, extraction time 360 minutes and frequency extraction 3 times give andrographolide content about 3.50%. While using the optimum extraction conditions from prediction profiles, using sample to solvent ratio 2g/100 mL, extraction time 360min and frequency 4 the andrographolide content ranged between 3.47-3.74%.

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