

# Antioxidant and Antidiabetic Activities of Ethanolic Citrus Hystrix Peel Extract: Optimization of Extraction Conditions

*by Aning Ayucitra*

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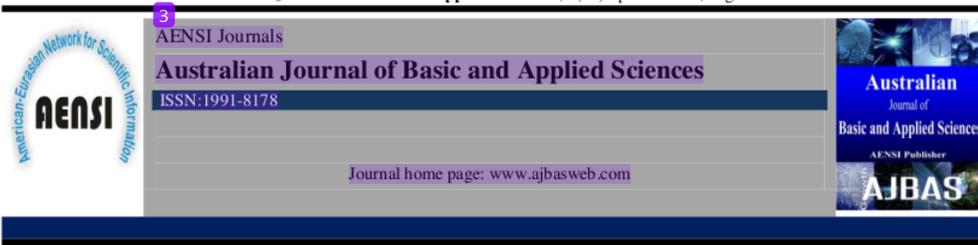
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## Antioxidant and Antidiabetic Activities of Ethanolic *Citrus Hystrix* Peel Extract: Optimization of Extraction Conditions

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### ABSTRACT

The benefits of citrus fruits have been well documented. As food ingredient, only leaf part of *Citrus hystrix* has been utilized as food ingredient. The citrus peel may contain higher antioxidant compounds than the edible part. The objective of this study was to optimize the extraction conditions of antioxidant, presented as Total Phenolic Compounds (RSM), from *Citrus hystrix* peel using Response Surface Methodology. In addition, *in-vitro* study to investigate the antidiabetic effect of the extract was also performed. Two independent variables (time and ethanol concentration) were optimized for maximizing the total phenolic compounds extracted from the *Citrus hystrix* peel using RSM based on central composite design. The optimum conditions of polyphenolic compounds extraction from *Citrus hystrix* peel were obtained at 7.8 h of extraction time by employing ethanol 41% as the solvent. The extract also exhibited the anti-diabetic activity.

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## INTRODUCTION

Citrus fruit has been known for its various health benefits. Polyphenolic compounds, a complex group of secondary metabolite substances, contains numerous biological active compounds, exhibit wide range of physiological properties such as anti-inflammatory (Menichini *et al.*, 2011), anti-microbial (Yi *et al.*, 2008), cardioprotective (Putri *et al.*, 2013), neuroprotective (Zbarsky *et al.*, 2005), anti-adipogenesis (Kim *et al.*, 2012), anti-diabetes (Li *et al.*, 2006), hepatoprotective (Putri *et al.*, 2013), etc. Various classes of polyphenolic compounds in citrus fruit have been identified as flavanone aglycones (hesperitin, naringenin), flavone aglycones (acacetin, quercetin, diosmetin), polymethoxyflavones (quercetogetin, nobiletin, tangeretin), flavanone-*O*-glycosides (hesperidin, naringin, narirutin, neohesperidin), and flavone-*C*- and flavone-*C*-glucosides (Gattuso *et al.*, 2007). The fruit peel has been reported to contain higher antioxidant compounds than the edible part (Gorinstein *et al.*, 2002) and therefore, study the antioxidant activity of the peel becomes a challenge. *Citrus hystrix* is one of citrus cultivars in Indonesia that has not been fully utilized yet because only leaves used as food ingredient. Therefore, it is an interesting challenge to investigate the antioxidant activity of Indonesian *Citrus hystrix* peel because its polyphenolic compounds would be different with the one growth in other areas as reported in literature. It opens opportunity to further develop Citrus peel as one of Indonesian natural antioxidant sources. There is no information related to antioxidant activity of Indonesian *Citrus hystrix* peel found in the literature. Moreover its antioxidant capacity as anti-diabetes was also another challenge to be investigated.

In order to extract the citrus fruit, maceration, hot water extraction, and reflux are commonly used procedures (Putri *et al.*, 2013; Xu *et al.*, 2008; Bocco *et al.*, 1998). The techniques are simple but consume large volume of solvents used. Supercritical fluid extraction, sub-critical water extraction, microwave-assisted extraction, and ultrasonic treatment are emerging as better alternatives than the former techniques (Ruen-ngam *et al.*, 2012; Cheigh *et al.*, 2012; Hayat *et al.*, 2010; Ma *et al.*, 2008). However, their high operating costs may have limited their small- or medium-scale industrial applications. In this study, maceration extraction was selected to extract antioxidant compounds from *Citrus hystrix* peel.

Several parameters influence the extraction of polyphenolic compounds such as extraction time, solvent-to-sample ratio, temperature, solvent type, and the number of repeat extraction. The optimum recovery of phenolic

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compounds would be different from one sample to others that depends on the nature or physicochemical properties of the samples. Response Surface Methodology (RSM) was employed to optimize of extraction process conditions of *Citrus hystrix* peel in this study. The method has been widely used for the optimization of extraction conditions such as temperature, solvent concentration, time, etc. RSM consists of mathematical and statistical techniques to develop a functional relationship between a response of interest and some independent variables.

This study was aimed to optimize the maceration extraction conditions of antioxidant (phenolic compounds) from *Citrus hystrix* peel using RSM. Additionally, the most important factor contribution to the antioxidant activity of the *Citrus hystrix* peel extract was determined. Anti-diabetes activity of the extract was also investigated.

## MATERIALS AND METHODS

Materials used in this study were *Citrus hystrix*, purchased from local market in Surabaya that were collected around January–March 2014. Ethanol (C<sub>2</sub>H<sub>5</sub>OH), aquades (H<sub>2</sub>O), Folin-Ciocalteu reagent, sodium carbonate (Na<sub>2</sub>CO<sub>3</sub>), gallic acid (C<sub>7</sub>H<sub>6</sub>O<sub>5</sub>), glucose (C<sub>6</sub>H<sub>12</sub>O<sub>6</sub>), dinitrosalicylic acid (C<sub>7</sub>H<sub>4</sub>N<sub>2</sub>O<sub>7</sub>), amyllum ((C<sub>6</sub>H<sub>10</sub>O<sub>5</sub>)<sub>x</sub>), enzyme  $\alpha$ -amylase (from *Aspergillus oryzae*) was purchased from suppliers and used without further purification.

Methods carried out involve the raw material preparation, extraction, and analysis steps. Firstly, fresh *Citrus hystrix* fruit were washed, peeled off and cut into a size of 0.5 x 0.5 cm prior to dry it for about 48 h at 35°C in an air-oven (Mentert, Germany). The material was then stored below 0°C in airtight plastic bags for further use. Secondly, the dried sample was weighed and extracted with 20 mL of solvent in a dark and closed container at room temperature and certain extraction time. After the extraction, the extract was separated and centrifuged to remove the solid part. Thirdly, the total phenolic content (TPC) of *Citrus hystrix* peel extract was determined using the Folin-Ciocalteu colorimetric method. The TPC value was expressed as gallic acid equivalents (GAE) per dry mass of *Citrus hystrix* peel sample (mg GAE/g) from a standard calibration curve. The extract was also tested for anti-diabetic activity by performing *in-vitro* study using enzyme  $\alpha$ -amylase as the selected model (Nair *et al.*, 2013).

Central composite design (CCD) was selected to determine the optimum condition of the extraction process by using MINITAB 14. CCD was constructed with four axial points, four star points ( $\pm \alpha$ ) and five replications at the center point. In this study, 1.41 was used as  $\alpha$  for rotatable design (Montgomery, 2005). The following regression equation was fitted to the response resulted from CCD by the least square method:

$$Y = \beta_0 + \sum_{i=1}^k \beta_i x_i + \sum_{i=1}^k \beta_{ii} x_i^2 + \sum_{i < j} \beta_{ij} x_i x_j \quad (1)$$

where Y is the estimated TPC yield,  $x_i$  and  $x_j$  are the coded value of an independent variables,  $\beta_0$  is a constant coefficient,  $\beta_i$  are linear coefficients,  $\beta_{ij}$  (i and j) are the interaction coefficients and  $\beta_{ii}$  are the quadratic coefficients.

## RESULTS AND DISCUSSION

In response surface methodology, natural variables are transformed into coded variables that have been defined as dimensionless with a mean zero and the same standard deviation. The experimental values for response (TPC) under different conditions were given in Table 1. Center point was set at 6 h of extraction time and 55% of ethanol concentration. In all experiments, solid-to-solvent ratio was fixed at 1:40 (w/v). The results showed that TPC of local *Citrus hystrix* peels ranged from around 30.02 to 63.19 mg GAE/g dry weight. By employing multiple regression analysis, the regression model related the selected independent variables and the response of TPC was follows:

$$Y = 58.370 + 6.979X_1 - 6.362X_1^2 - 3.863 X_1^2 - 3.393 X_2^2 + 2.143 X_1X_2 \quad (2)$$

where Y, X<sub>1</sub>, and X<sub>2</sub> are TPC, extraction time, and ethanol concentration, respectively.

The fitness and adequacy of the model was further evaluated by the determination coefficient (R-sq). The R-sq, defined as the ratio of the explained variation to the total variation, was a measure of the degree of fit. The closer the R-sq value, the better the empirical model fits the experimental data. The R-sq of the work shown in Table 1 indicates that 89.2% of the total variation around the average could be explained by the regression model. This suggests that the predicted second order polynomial model defined quite well the real behaviour of the system.

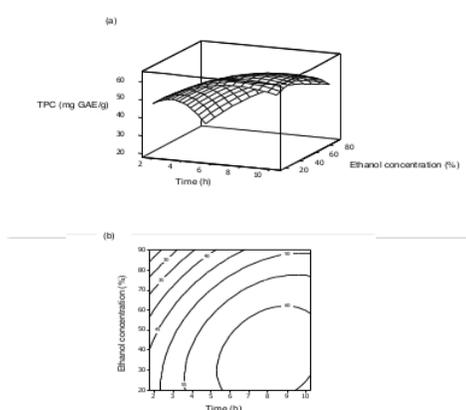
Figure 1(a, b) illustrates three-dimensional response surface plot by presenting the response of TPC as a function of the two selected variables. As seen, higher amount of phenolic content extracted from the peel in the region of extraction time between 7 to 8.5 h and ethanol concentration between 30 and 45%. Therefore, the yield of TPC continues to increase with ethanol concentration and extraction time and achieved the optimum conditions at about 7.8 h and 41%, respectively, before it began to decrease. The predictive TPC value at these conditions is 62.22 mg GAE/mg. Generally, the polarity of ethanol-water mixture increases continuously with

the water addition into the system. The optimum ethanol concentration (41%) observed in this work indicates that *Citrus hystrix* peel contains more polar compounds. Polar phenolic compounds reported in citrus are aglycones and glycosides of flavonoids, neohesperidin and neorocitrin (Chan *et al.*, 2009). The increase of TPC yield with extraction time can be explained by the longer the peel was soaked in the solvent, the longer the contact between the two and thus, more compounds in the solid part will be dissolved which the amount and the extraction rate are depend on the solvent polarity. Further verification of the predictive model was performed by repeating the experiment at the optimum conditions and the observed TPC yield was 64.24 mg GAE/mg. This result indicates that the experimental result was quite close to the predicted one, indicating the high fit degree between the observed experiment and the response predicted from the regression model.

**Table 1:** Independent variables and experimental TPC yields of the CCD.

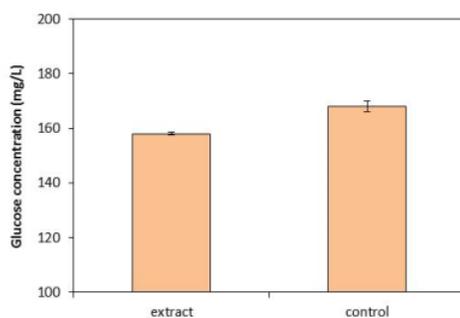
Run	Coded variables		Real variables		TPC (mg GAE/g)
	X <sub>1</sub>	X <sub>2</sub>	X <sub>1</sub>	X <sub>2</sub>	
1	-1	-1	3	30	52.50
2	-1	+1	3	80	30.02
3	+1	-1	9	30	63.19
4	+1	+1	9	80	49.28
5	0	0	6	55	57.68
6	0	0	6	55	59.52
7	0	0	6	55	57.85
8	0	0	6	55	58.32
9	0	0	6	55	58.48
10	-1.41	0	1.8	55	43.86
11	+1.41	0	10	55	62.16
12	0	-1.41	6	20	59.08
13	0	+1.41	6	90	48.82

X<sub>1</sub> and X<sub>2</sub> represent extraction time (h) and ethanol concentration (%), respectively. R-sq = 0.892.



**Fig. 1:** (a) Response of TPC and (b) contour plot of TPC calculated from the model.

The extract of *Citrus hystrix* peel was also subjected for anti-diabetic activity test. The study on anti-diabetes from natural resources becomes interest because long-term changes, dysfunction and failure of organs after consuming modern medicines for a period of time. In addition, Indonesia is the fourth country after India, China, and United States with the largest number of diabetic patients. The important aim to treat diabetic patients is by stabilizing blood glucose level. Glucose or starch absorption can be eliminated by consuming inhibitor of carbohydrate digestion such as  $\alpha$ -amylase and  $\alpha$ -glucosidase inhibitors. The inhibitor will prevent polysaccharides and/or disaccharides from being hydrolyzed to monosaccharides in small intestine. In this work,  $\alpha$ -amylase was employed as the inhibitor. Figure 2 shows anti-diabetic activity of the extract of *Citrus hystrix* peel. As seen, the extract inhibits the conversion of starch to glucose as shown by lower glucose concentration observed in solution than the control one. This can be explained by the presence of polyphenolic compounds (TPC) in the extract promote its anti-diabetic activity. The presence of gallic acid, hesperidin, and naringin in citrus fruits have been suggested to be responsible for this (Patel and Goyal, 2011; Jung *et al.*, 2004).



**Fig. 2:** Effect of the addition of *Citrus hystrix* peel extract on glucose production.

#### Conclusions:

In the present study, the extraction of total phenolic compounds from *Citrus hystrix* peel was optimized using response surface methodology based on central composite design. The quadratic model for predicting the total phenolic content was obtained and both factors investigated here contributed significantly to the TPC yield. The optimum extraction conditions of extraction time and ethanol concentration were 7.8 h and 41%, respectively. The most important result observed here is that *Citrus hystrix* peel extract also exhibited anti-diabetic activity. Further study needs to be investigated to get further insight on what specific phenolic compound was responsible for this activity.

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