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OPTIMIZATION OF PHENOLIC COMPOUNDS EXTRACTION FROM *Averrhoa bilimbi* AND *Citrus hystrix* PEEL USING STATISTICAL DESIGN OF EXPERIMENT

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ABSTRACT

In this study, optimization of *Averrhoa bilimbi* and *Citrus hystrix* extraction using maceration extraction methods was performed using a statistical method design of experiment (DOE). A two factorial design of experiment was performed with ethanol concentration and ratio of solid to liquid as the controlled variables. Results showed that an optimized maceration extraction method of phenolic compounds from *Averrhoa bilimbi* and *Citrus hystrix* peel has been developed. The highest TPC value of *Averrhoa bilimbi* crude extract, i.e. 5.45 mg GAE/g *Averrhoa bilimbi* powder was obtained at the following maceration condition: ratio solid to liquid of 1 to 15 using ethanol with concentration of 48% as solvent. Whilst, for citrus peel crude extract, the highest TPC value was 55.71 mg GAE/g dried citrus peel under the following maceration condition: ratio solid to liquid of 1 to 50 using aquadest as solvent. From DOE analysis, it is known that controlled variables used have significant effects to TPC as response, even though there was no significance interaction between variables.

Keywords: phenolics, extraction, optimization, *Averrhoa bilimbi, Citrus hystrix*

INTRODUCTION

Averrhoa bilimbi and *Citrus hystrix,* or locally known as *belimbing wuluh* and *jerukpurut*, respectively, are two common plants used as traditional cooking ingredients in Indonesia. Both easily grow in almost part of Indonesia especially in Java Island. Despite their sourness and as fragrance for cooking, several studies have found that *Averrhoa bilimbi* and *Citrus hystrix* comprise high amount of bioactive compounds (Laohavechvanich *et al*., 2010; Chowdhury *et al*., 2012; Hasanuzzaman *et al*., 2013). Composition of bioactive compounds varies greatly between varieties.

Antioxidative compounds in *Averrhoa bilimbi* may be used as natural antioxidant, anti-aging, anti-inflammation, and anti-cancer.Studies conducted by Chowdhury *et al*. (2012) dan Hasanuzzaman *et al*. (2013) have shown that methanolic extracts of *Averrhoa bilimbi* fruits contain alcaloid, tannin, saponins, and flavonoids. Chowdhury *et al*. (2012) obtained total phenolic content (TPC) of 106.16 mg Gallic Acid Equivalent (GAE) per g *Averrhoabilimbi* fruit extracts dan total flavonoid content (TFC) of 276.73 mg Quercetine Equivalent (QE) per g *Averrhoabilimbi* fruit extracts from a soxhlet extraction method at elevated temperature of 65ºC using 70% methanol as solvent. Study by Hasanuzzaman *et al*. (2013) showed a different result.They used maceration method in methanol for 15 days at room temperature. As results, TPC of extracts were in the range of 50.23-68.67 mg GAE/g extracts with IC_{50}value of 30.365 µg/mL methanol. These differences demonstrate that method of extraction and temperature during extraction process affect the value of total phenolic content of extracts. In this case, soxhlet extraction method and elevated temperature give higher value of TPC. In addition, a study by Kuncahyo and Sunardi (2007) revealed that phenolic compounds of *Averrhoa bilimbi* tends to easily extracted in polar solvents such as water than in ether.

Peel waste contains high level of antioxidative compounds (Li *et al*., 2006; Zia-ur-Rehman, 2006; Xu*et al*., 2008; Nam *et al*., 2009; Wang *et al*., 2011), which could be higher than the fruits. Studies have shown that *citrus* peel could be a potential source for natural antioxidant due to its high content of phenolic and flavonoid compounds. There are three types of flavonoid compounds generally found on *citrus* peel; *flavanone* (*e.g.* hesperidin, naringin, and hesperitin), *polymethoxylated flavone* (*e.g.*nobiletin dan tangeretin), and *flavonol* (*e.g.* rutin) (Choi *et al*., 2007). In general, *flavanone* is the major flavonoid constituents in *citrus* peel with hesperidin and/or naringin as the most dominant depending on the source and type of citrus studied (Peterson *et al*., 2006; Choi *et al*., 2007).

Study on antioxidative compound extraction from *Citrus* peel using different types of solvents and polarity levels had been conducted by some researchers (Chan *et al*., 2009; González-Molina *et al*., 2010). Chan *et al*. (2009) compared three different types of solvent namely ethanol, methanol, and acetone during *Citrus hystrix* peel extraction and reported that types of solvent extraction affected the yield of phenolic of extracts. Ethanol as solvent gave the best result. However, neither total flavonoid content analysis of extracts nor bioactive compound identification was reported by Chan *et al*. (2009). Hence, fundamental theory of which bioactive compounds inside *Citrus hystrix* peel responsible for antioxidant activity is not yet fully understands.

Previous studies have shown that extraction efficiency relied on following parameters: plant source, different types of organic solvents, solvent concentration, and extraction temperature. Studies on *Averrhoa*

*bilimbi*and *Citrus hystrix* peel extraction gave various results. Moreover, the advantages of extracts obtained from local *Averrhoa bilimbi*and *Citrus hystrix* peel still remains unexplored. The objective of this study was, therefore, to evaluate the effects of extraction condition, namely type of solvent, and ratio of solid to solvent, on total phenolic content of crude extracts obtained from *Averrhoa bilimbi* and *Citrus hystrix* peel. Significance of variables and interaction between variables in response to total phenolic content was also studied using a statistical full factorial design of experiment.

MATERIALS AND METHODS

Materials and equipment

Averrhoa bilimbi fruits were collected from traditional market in Surabaya - East Java, whilst for *Citrus hystrix*were collected from Semarang - Central Java, Indonesia.Folin-Ciocalteure agent (Merck, Germany),gallic acid standard (Sigma-Aldrich, USA), and other chemicals were obtained from local distributors. All solvents and chemicals used were of analytical grade and used without further treatment.

Phenolics extraction

Polyphenolic compounds were extracted from *Averrhoa bilimbi* fruits and *Citrus hystrix* peel using maceration method. Independent variables evaluated were ethanol concentrations (*i.e.* 0, 48, and 96%) and solid to liquid ratio (*i.e.* 1:5, 1:10, 1:15 for *Averrhoa bilimbi* and 1:30, 1:40, 1:50 for *Citrus hystrix* peel). *Averrhoa bilimbi* fruits were firstly washed, cut, and oven-dried at 40ºC for 5 days. The dried fruits were then crushed into powder, sieved, and stored until further used. The dried fruit powder had moisture content of 7-8%. As for *Citrus hystrix*, the peel was cut into 0.5 x 0.5 x 0.1 in size and oven-dried at 40ºC for 3 days until its moisture content reached 7-8%. The dried peel was stored until further used.

Each material was extracted with ethanol at various concentrations. The maceration extraction was conducted for 24 h at room temperature. Following extraction, the crude extract (filtrate) was separated from the solid residues using Buchner separator. The crude extract was evaporated in a rotary evaporator (IKA, RV-10) below 40ºC and further dried in a vacuum oven (Vacuum Lab-Line, USA). The dried extracts were used as natural antioxidant. All experiment was performed in triplicate.

Total phenolic content determination of crude extracts

The concentration of total phenolic compound of extracts was determined spectrophotometrically using the Folin–Ciocalteu total phenol procedure described by Waterhouse (1999), with minor modifications. Gallic acid standard solutions were prepared at 50, 100, 150, 200, and 250 mg/L. One millilitre of crude extracts and 1 mL of gallic acid standard (Sigma-Aldrich, USA) were transferred into separate 15 mL test tubes. Five millilitre of 0.2 N Folin-Ciocalteureagent (Merck, Germany) were added to each test tube and mixed. After 1 min, 4.0 mL of 7.5% (w/v) sodium carbonate solution were added and mixed. Prior to measurement, every sample solution was left for 30 min at room temperature. The absorbance of samples was then measured spectrophotometrically at 736 nm using a Shimadzu UV-VIS 1700 spectrophotometer. A calibration curve of gallic acid was plotted by plotting absorbance versus gallic acid concentrations (mg/L). Total phenolic compounds concentration in the crude extracts was determined by comparing the absorbance of the extract samples to that of gallic acid standard solutions. All samples were analyzed in duplicate. Total phenolic content of samples was expressed as milligram gallic acid equivalents (GAE) per g dried raw material.

A Full factorial design of experiment

Evaluation of significant levels of each variables and effect of interaction between variables during maceration extraction in response to total phenolic content of extracts was carried out using a full factorial design of experiment (DOE). This kind of analysis aims to determine the significance of variables used in an experiment, which is beneficial for process optimization. In this study, the DOE was performed using a Minitab software (Minitab Version 15.1.1.0.). A central composite design was used to investigate the effects of two controlled variables at three levels. Controlled variables and their levels used throughout this study are presented in Tables 1 and 2 for *Averrhoa bilimbi* and *Citrus hystrix* peel, respectively. The adequacy of the model was determined by evaluating the lack of fit and coefficient of determination generated by the software.

Table-1. The controlled variables for DOE and their levels for *Averrhoa bilimbi*extraction.

Parameters	Low level (-1)	Centre point (0)	High level (+1)
Ethanol concentration, %w (A)	0	48	96
Solid to liquid ratio, w/v (B)	1:5	1:10	1:15

Table-2. The controlled variables for DOE and their levels for *Citrus hystrix* peel extraction.

Parameters	Low level (-1)	Centre point (0)	High level (+1)
Ethanol concentration, %w (A)	0	48	96
Solid to liquid ratio, w/v (B)	1:30	1:40	1:50

RESULTS AND DISCUSSIONS

Effect of solid to liquid ratio and ethanol concentration on total phenolic content (TPC)

Total phenolic content (TPC) of *Averrhoa bilimbi* and *Citrus hystrix* peel crude extracts at various solid to liquid ratio and ethanol concentration is presented in Figure-1 and Figure-2, respectively. Total phenolic content of *Averrhoa bilimbi* crude extracts was in the range of 2.92 to 5.45 mg GAE/g dried *Averrhoa bilimbi*, whilst for *Citrus hystrix* peel, the range of TPC was 10 folds higher *i.e.* 27.56 to 55.71 mg GAE/g dried *Citrus hystrix* peel.

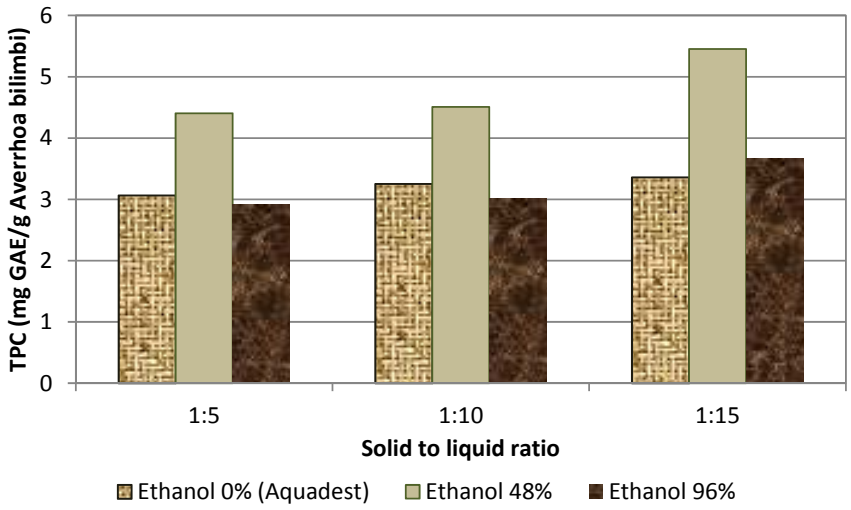


Figure-1. TPC of *Averrhoa bilimbi* crude extract at various solid to liquid ratio and ethanol concentration (in mg GAE/g dried *Averrhoa bilimbi*).

Figure-1 shows that for the same solid to liquid ratio, maceration extraction with ethanol concentration of 48% resulted *Averrhoa bilimbi* crude extracts with the highest total phenolic content. Phenolic/flavonoid compounds such as p-hydroxybenzoic acid, chlorogenic acid, p-coumaric acid, ferulic acid, resveratrol, and quercetin have higher solubility in ethanol than in water, whilst epicatechin possesses higher solubility in water. Solvent ability in extracting phenolic/flavonoid compounds from natural resources depends on its polarity. Amount of water in ethanol solution affects the level of polarity; ethanol is less polar than water due to its covalent bonding. Different in polarity of solvents used will give crude extracts with different composition of phenolics. Previous study showed that types of solvent used affected the extraction process because each solvent possessed different level of polarity and thus selectivity to different bioactive compounds (Li *et al*., 2006; Zia-ur-Rehman, 2006; Jain *et al*., 2009). Solvent level of polarity can also be seen from its dielectric constant. The higher the dielectric constant of a solvent, the higher the level of polarity. Water has higher dielectric constant (80) compared to that of ethanol (30) (Jain dkk, 2009). In this study, the highest level of TPC (5.45 mg GAE/g dried *Averrhoa bilimbi*) was obtained by maceration extraction at the following condition: solid to liquid ratio of 1 to 15 and ethanol concentration of 48%.

Citrus hystrix peel extraction gave different result compared to that of *Averrhoa bilimbi*, as shown in Figure 2. TPC of *Citrus hystrix* peel crude extracts were about 10 folds higher than that of *Averrhoa bilimbi*. The highest TPC value was 55.71 mg GAE/g dried *Citrus hystrix* peel which was obtained from maceration extraction with solid to liquid ratio of 1 to 50 and aquadest as solvent. Extraction using ethanol 0% or aquadest as solvent gave better crude extracts with regards to total phenolic content, for all range of solid to liquid ratio studied.

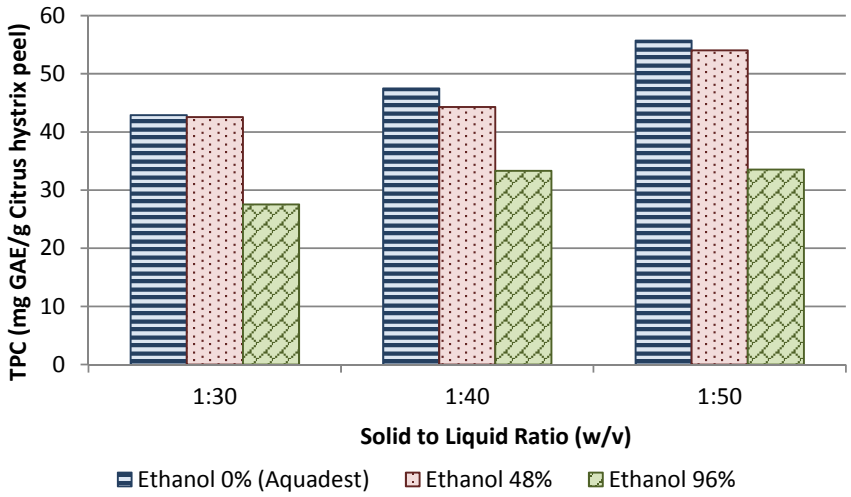


Figure-2. TPC of *Citrus hystrix* peel c*rude* extract at various solid to liquid ratio and ethanol concentration(in mg GAE/g *Citrus hystrix* peel).

Different level of polarity of solvents used in *Citrus* peel extraction also give extracts with various phenolics/flavonoids composition (Zia-ur-Rehman, 2006; Chan *et al*., 2009). *Citrus hystrix* peel crude extracts in this present study contained gallic acid, rutin, naringin, hesperidin, and naringenin. This result was in accordance to the previous study conducted by Petersone*t al*. (2006) and Choi *et al*. (2007).For both materials, the greater the solid to liquid ratio, TPC on crude extracts obtained was higher. By using greater amount of solvent, the concentration gradient of phenolics between solid and solvent was higher, therefore, the mass transfer between the two as driving force was also higher (Li *et al*., 2009; Baiano *et al*., 2014).

Analysis of main effect plot

Main effect plot illustrates the increase or decrease in the value of the response (TPC) for each variable. Results of main effect plot analyses can be seen in Figures-3 and 4, for *Averrhoa bilimbi* and *Citrus hystrix* peel, respectively. Based on the figures, the black line showed the increase or decrease in response (TPC) for each variable that is of a lower level (-1), center point level (0), and upper level (+1). As shown in Figure-3, TPC of *Averrhoa bilimbi* crude extract will be greater with the increasing solid to liquid ratio, from 1:5 (level -1) to 1:15 (level +1). On the other hand, the increased in ethanol concentration from 48% (level 0) to 96% (level 1) has reduced TPC value of extracts. Ethanol concentration of 48% gave the greatest value of TPC amongst all ratios. This was consistent with the extraction results in Figure-1.

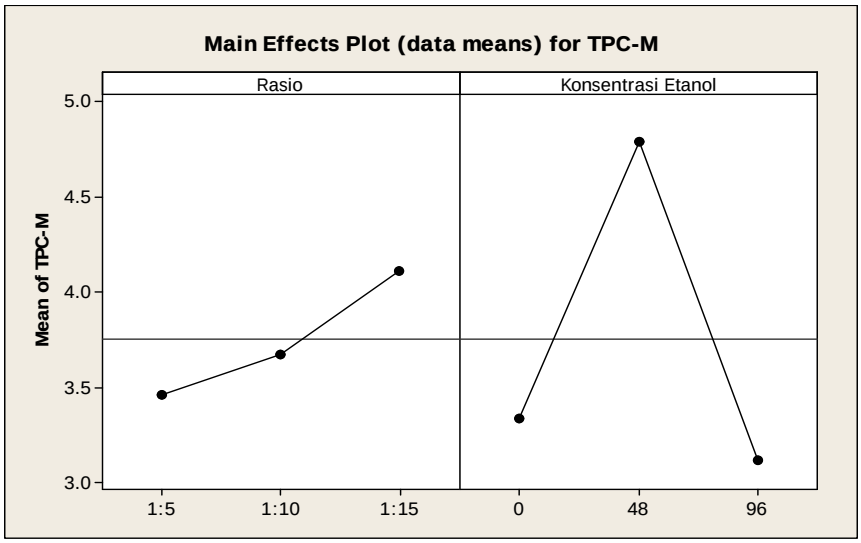


Figure-3. Main effect plot on TPC response of *Averrhoa bilimbi* crude extract.

Similar thing was also noticed for *Citrus hystrix* peel results, shown in Figure-4. TPC of the crude extracts will be greater with the increasing solid to liquid ratio, from 1:30 (level -1) to 1:50 (level +1). However, there was no optimum condition observed from Figure-4 in response to TPC.

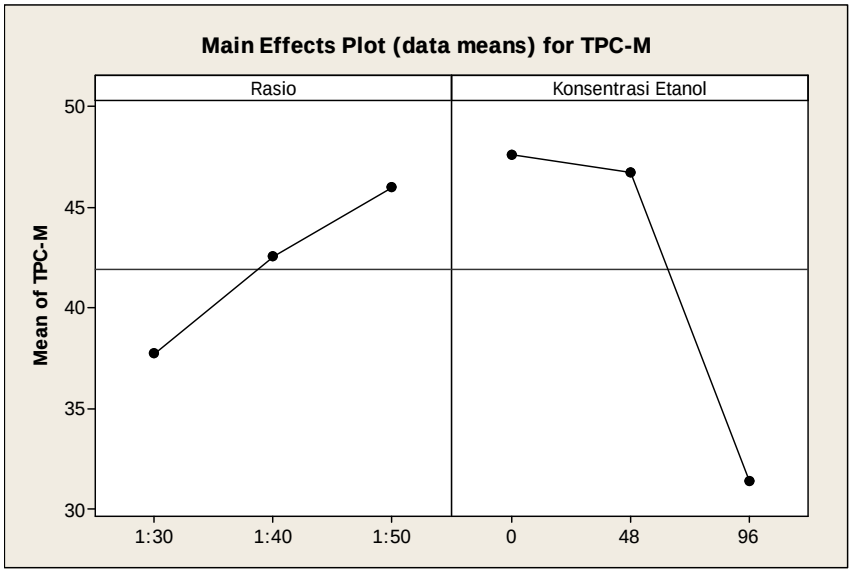
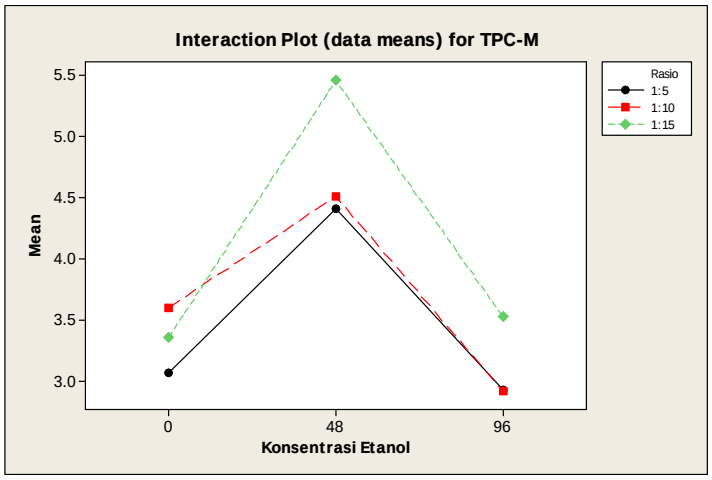


Figure-4. Main effect plot on TPC response of *Citrus hystrix* peel crude extract.

Analysis of interactions plot

Interactions plot describes the interaction between two variables where each variable has an influence on other variables in producing TPC response. Interaction plots of this present study are shown in Figure 5 and 6. The figures show the interaction between controlled variables used namely solid to liquid ratio and ethanol concentration. The black line shows solid to liquid ratio of 1 to 5 (level -1), the red line indicates the solid to liquid ratio of 1 to 10 (level 0), and the green line shows the solid to liquid ratio of 1 to 15 (level +1). From both figures, it can be concluded that interaction between controlled variables gave insignificant effect on TPC. It means that there was no significant effect on response to TPC when both controlled variables are being used at the same time. This result is confirmed with the p-value obtained for interaction between ratio and ethanol concentration that is > 0.05. Amongst all controlled variables, solid to liquid ratio gave most significant effect on the increase in total phenolic content of both crude extracts.

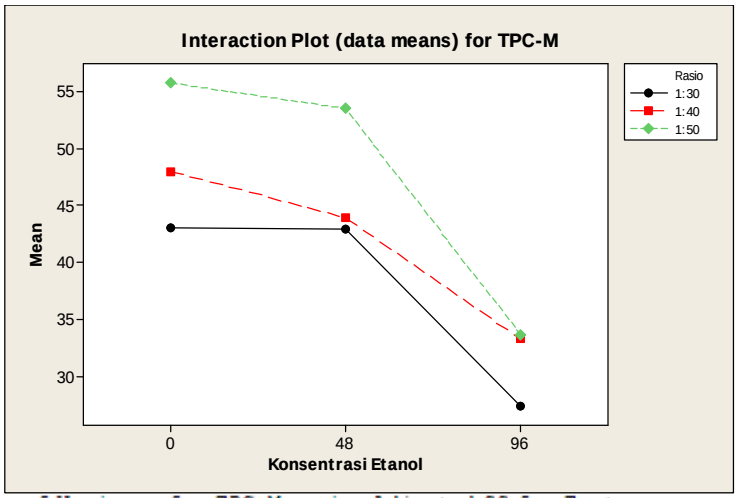


Analysis of Variance for TPC-M, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Ratio	2	1.3142	1.3142	0.6571	4.15	0.053
Ethanol Concentration	2	9.8271	9.8271	4.9136	31.02	0.000
Ratio*Ethanol Concentration	4	0.8035	0.8035	0.2009	1.27	0.351
Error	9	1.4254	1.4254	0.1584		
Total	17	13.3702				

S = 0.397970 R-Sq = 89.34% R-Sq(adj) = 79.86%

Figure 5. Interaction plot with response to TPC for *Averrhoa bilimbi* crude extract.



Analysis of Variance for TPC-M, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Ratio	2	189.02	248.99	124.49	547.26	0.000
Ethanol Concentration	2	1047.75	1039.88	519.94	2285.62	0.000
Ratio*Ethanol Concentration	4	50.87	50.87	12.72	55.91	0.000
Error	9	2.05	2.05	0.23		
Total	17	1289.69				

S = 0.476952 R-Sq = 99.84% R-Sq(adj) = 99.70%

Figure-6. Interaction plot with response to TPC for *Citrus hystrix* peel crude extract.

CONCLUSIONS

Results showed that extraction condition affected the total phenolic content of crude extracts obtained from *Citrus hystrix* peel and *Averrhoa bilimbi*. The highest total phenolic content of crude extracts obtained were 55.71 mg GAE/g *Citrus hystrix* peel and 5.45 mg GAE/g *Averrhoa bilimbi*. From statistical analysis, it was known that control variables chosen had significant effect in response to total phenolic content even though there were no significant interactions between variables. Amongst variables, solid to liquid ratio gave most significant effect on the increase in total phenolic content of both crude extracts.

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