Analysis of antioxidant property of the extract of saponin by experiment design methodology

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Abstract. The antioxidant properties of the extract of saponin from Sapindus mukorossi (soapberry) were analyzed and optimized using Response Surface Methodology (RSM). Based on this study, by using different methods to evaluate its antioxidant properties for further analysis. The methods are 1,1-di-phenyl-2-picryl-hydrazil (DPPH) free radical scavenging assay, reducing power, and ferrous ion chelating ability. The best DPPH free radical scavenging ability was obtained at 91.56 % (X1 = 1 hour, X2 = 1:15 (w/v), X3 = 0.55 %), in which, the influences of solid to liquid ratio (X2) and enzyme concentration (X3) was found to be significant on the response of percentage of free radical scavenging effect (Y1). The best reducing power was obtained in Absorbance (Abs) 0.473 (X1 = 3 hour, X2 = 1:10 (w/v), X3 = 0.1 %), in which, the influences of solid to liquid ratio (X2) was found to be significant on the response of amount of reducing power (Y2). The best ferrous ion chelating ability was obtained at 49.63 % (X1 = 1 hour, X2 = 1:10 (w/v), X3 = 1 %), in which, the influence of time (X1) and solid to liquid ratio (X2) was significant on the response of percentage of ferrous ion chelating ability (Y3). The extract of saponin from Sapindus mukorossi shows excellent in antioxidant capacity based on the reducing power and scavenging free radicals activity, but it is relatively weak in ferrous ion chelating.

1. Introduction
Many antioxidant compounds from plants have been reported as active oxygen or free radical scavengers. This has gained interest to find natural antioxidant for nutraceutical application in order to substitute synthetic antioxidant, which cause many side effects such as carcinogenicity. The natural antioxidant can inhibit the process of oxidizing chain reactions and keep the human bodies from free radical attack which causing many chronic illness [1-2].

Saponins are surface-active glycosides and are commonly found in plants. It consists of a sugar moiety linked to aglycone which may be triterpenoid or steroid. Its diverse compound structurally has been also observed to be antioxidant. The mechanism of its antioxidant for preventing the bio-molecular damage that caused by free radicals, is by configuring hydroperoxide intermediates [3]. The rich of saponins can be found in the pericarp of Chinese soapberry tree (Sapindus mukorossi) [4]. In general, this plant is used as commercial cleaner such as shampoo and traditionally used for removing lice from scalp. It was reported that this plant has revealed many pharmacological activity [5]. Moreover, the study of S.
mukorossi extracts show many biological activities beside antioxidant, such as anti-tyrosinase, antimicroorganism and anticancer. Thus, it displays high potential for application of S. mukorossi extracts in many kinds of field such as cosmetic, food supplementation, antibiotics and medical application [6]. To obtain saponin from this plant, some methods have been conducted by using organic solvent such as methanol [7]. Even though it has a high effectiveness to extract saponin after acetone and also has shown high antioxidant activity [8-9], but unfortunately it is harmful and constitutes the health risk for the environment. Another method to obtain high antioxidant property is by using the enzyme. The enzyme is highly efficient and accurate, and can cause different degrees of changes in the plant cell wall, such as breaking down and building up, therefore by changing the permeability of the cell wall, and improving the extraction rate [10].

Regarding to the importance of extraction as the first step in the purification of active ingredients as product from plant material, the extraction methods need to be developed and moreover, many factors are contributed in the process such as solid to liquid ratio, temperature, particle size, etc. Optimization is one of the ways to develop the process in order to improve the quality of product with more efficient and less time consuming by using experimental design. Response surface methodology (RSM) is platform of Design of Experiment and a collection of mathematical and statistical techniques that may be useful in the development, improvement and formulation of [11].

The objective of this study is to analyze the antioxidant properties of the extracted saponins using enzyme extraction. Further, using RSM, some factors, such as solid to liquid ratio, concentration of enzyme, and hydrolysis time that affect the antioxidant properties from the extract of saponin, can be known which one is the optimum condition and the significant factors that influences the response. Moreover, the result of the enzymatic extraction process from those factors can be expressed with the mathematical model. This model is able to control, preserve it at optimal level, give highest yield, and attain the result at lowest cost [12].

2. Experiment

2.1. Chemicals required
Potassium hexacyanoferrate (III), sodium dihydrogenphosphate anhydrous (NaH$_2$PO$_4$) and Disodium hydrogenphosphate (Na$_2$HPO$_4$) were purchased from Showa chemical co. ltd, Japan. Iron (III) chloride hexahydrate 99% and Iron (II) chloride tetrahydrate were purchased from Sigma-Aldrich (St. Louis, USA), Trichloroacetic acid 99% was purchased from Acros organics (Germany), 5,6-Diphenyl-3-(2-pyridyl)-1,2,4-triazine-4,4”-disulfonic acid monosodium salt hydrate 97% (ferrozine) was purchased from Alfa Aesar (Great Britain) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) (free radical) 95% was purchased from Alfa Aesar (Germany).

2.2. Extraction of saponin from fruit of S. mukorossi
The fruit of S. mukorossi was vacuum freeze dried product and it was purchased from True Ten Industrial Co. Ltd, Taichung, Taiwan. The dried fruit was grinded into 100 mesh. Conventional extraction method using deionized water as solvent was performed with different solid liquid ratio at 50°C. An electromagnetic heating stirrer (Comming, PC-420D) was used at 550 rpm of stirring speed. Cellulase onozuka R-10 from Trichoderma viride was purchased from Duchefa biochemie, Netherlands, was added when the temperature already at 50°C to start the extraction process. The supernatant was obtained by centrifugation in a centrifuge machine (EPPENDORF, 5415R) for 10 minutes at 3000 rpm. Extracted sample were stored at 4°C for antioxidant analysis. Extracted saponin sample was used to analyze about the antioxidant property using different method such as DPPH scavenging effect, reducing power, and ferrous ion chelating ability. Every sample need to be diluted into 1 mg/ml of saponin concentration.
2.3. DPPH scavenging assay measurement

DPPH scavenging effect was conducted using modified method from Shimada et al 1992 [13]. The formula to calculate DPPH scavenging effect is written below,

\[
\text{Scavenging effect (\%)} = \left(1 - \frac{\text{Sample}_{517} - \text{Blank}_{517}}{\text{Control}_{517}}\right) \times 100\%
\]

Sample: 0.1mM of DPPH was added 3 mL into the 6 mL of diluted sample; Blank: 6 mL of diluted sample was added into 3 mL of deionized water (DI water); Control: 4 mL of DI water was added into 2 mL of DPPH solution. For sample and control, we need to wait for 30 minutes in dark condition in order to avoid decomposition of DPPH solution because DPPH is sensitive with the light. All of them will be measured in the UV-spectrophotometer at wavelength 517 nm.

2.4. Reducing power measurement

This antioxidant activity was evaluated according to Oyaizu (1986) [14] method with some modifications. The diluted sample was added by 2 mL of Phosphate buffer solution (0.2 M PBS) and 2 mL of 1% of Potassium hexacyanoferrate. The mixture was incubated for 20 minutes in the hot water bath at 50°C. Then, 2 mL of 10% trichloro acetic acid was added into the mixture and centrifuge it for 10 minutes at 5000 rpm. After that, 1.5 mL of supernatant was taken to be added by 1.5 mL of DI water and 0.3 mL of iron chloride, then incubated for 10 minutes in dark condition. Measurement was performed at 700 nm. For the control, it just substitutes the sample with DI water. The formula to calculate the reducing power is written below,

\[
\text{Reducing power} = \text{Absorbance value of Sample} - \text{Absorbance value of control}
\]

2.5. Ferrous ion chelating measurement

This measurement was conducted using Dinis et al. (1994) [15] with some modifications. The ferrous ion chelating can be calculated using this formula.

\[
\text{Percentage of inhibition ferrozine} - \text{Fe}^{2+} (\%) = \left(1 - \frac{\text{Sample}_{562} - \text{Blank}_{562}}{\text{Control}_{562}}\right) \times 100\%
\]

Sample: 3 mL of diluted sample was added 0.15 mL of FeCl$_2$ then wait for 30 s for reaction, after that add 0.3 mL of ferrozine and wait for 10 minutes in dark condition; Blank : 3 mL of diluted sample was added 0.45 mL of DI water; Control : the procedure was the same like the sample, only substitute the diluted sample with DI water. All of them were measured at 562 nm using UV spectrophotometer.

2.6. Selection factors and statistical analysis

In this study with three factors and three levels, as shown in Table 1. Previous work [16] that have been conducted in quantifying saponin concentration through a one-factor test, only one factor is moved at a time, while other factors maintain at the level of the previous experiment to analyses the influence of factor level shifting. Thus, the selection of the optimum factor level ranges from each factor are carried out to find the high saponin concentration. The factors were chosen which $X_1$ as hydrolysis time, $X_2$ as solid to liquid ratio, and $X_3$ as concentration of enzyme. The response from the factors were antioxidant properties, such as the DPPH effect ($Y_1$), reducing power ($Y_2$), and ferrous ion chelating ability ($Y_3$). The three levels of variables in this study was shown in table 1 and it used Box behken design to run the experiment. All of the above experiments were conducted triplicate. Data was analyzed as mean by using statistical software, Response Surface Design JMP version 13.0.0 (statistical discovery from SAS, USA)
in which many analysis platforms were provided. One-way analysis was used to examine relationship between Y (response) and X (variables) and for additional analysis to convince the result, Analysis of Variance (ANOVA) was applied to test the significance (p≤0.05) by t-test. From ANOVA, the relation between variables and the influence of each factors were to be identified.

Table 1. The selection of three factors with the levels for design experiment

| Level | X₁ (hour) | X₂ (w/v) | X₃ (%) |
|-------|-----------|----------|--------|
| -1    | 1         | 5        | 0.1    |
| 0     | 2         | 10       | 0.55   |
| +1    | 3         | 15       | 1.0    |

3. Result and discussion

3.1. DPPH free radical scavenging ability

Experimental design provided 15 experimental runs as shown in Table 2 for extraction process using enzyme in which the response of DPPH effect (Y₁) were in the list. The 9th run achieved highest result was attained at 91.56 % in the operating condition of time (X₁) at 1 hours, solid to liquid ratio (X₂) at 1:15, and enzyme concentration (X₃) at 0.55 %.

Table 2. The responses of Y₁, Y₂ and Y₃ in the Box Behnken experimental runs design.

| Run | Mode | X₁ | X₂ | X₃ | Y₁ (%) | Y₂ (Abs) | Y₃ (%) |
|-----|------|----|----|----|--------|----------|--------|
| 1   | -0+  | 1  | 10 | 1  | 87.66  | 0.380    | 49.63  |
| 2   | 000  | 2  | 10 | 0.55 | 82.47  | 0.419    | 41.94  |
| 3   | 0--  | 2  | 15 | 0.1 | 82.25  | 0.404    | 47.85  |
| 4   | −−0  | 1  | 5  | 0.55 | 70.37  | 0.208    | 42.29  |
| 5   | 0+−  | 2  | 5  | 1  | 69.70  | 0.238    | 44.62  |
| 6   | 000  | 2  | 10 | 0.55 | 81.60  | 0.468    | 38.89  |
| 7   | +0−  | 3  | 10 | 0.1 | 84.52  | 0.473    | 44.09  |
| 8   | −0−  | 1  | 10 | 0.1 | 81.17  | 0.439    | 47.31  |
| 9   | −+0  | 1  | 15 | 0.55 | 91.56  | 0.397    | 48.39  |
| 10  | ++0  | 3  | 15 | 0.55 | 90.48  | 0.381    | 45.16  |
| 11  | 0++  | 2  | 15 | 1  | 90.04  | 0.368    | 47.16  |
| 12  | 0−+  | 2  | 5  | 0.1 | 79.65  | 0.231    | 42.11  |
| 13  | +0+  | 3  | 10 | 1  | 84.09  | 0.359    | 48.03  |
| 14  | −0+  | 3  | 5  | 0.55 | 76.95  | 0.247    | 39.17  |
| 15  | 000  | 2  | 10 | 0.55 | 81.60  | 0.432    | 40.86  |

Based on the 15 experimental runs with the factors of Y₁, a second-order polynomial equation was obtained by the regression and it expressed in this following equation (1).
The plot which was drawn between the experimental data of \( Y_1 \) and the predictions result from Equation 1 shows on Figure 1. Based on the plot, it indicated that almost all of predictions data are fit with the result data from the experiment. Moreover, it was convinced by \( R^2 \) value that reached 0.95.

\[
Y_1 = 81.89 + 0.66 \times (X1 - 2) + (7.207) \times \left( \frac{X2 - 10}{5} \right) + (0.487) \times \left( \frac{X1 - 0.55}{0.45} \right) + (X1 - 2) \times \left( \frac{X2 - 10}{5} \right) \times (-1.915)
+ (X1 - 2) \times \left( \frac{X3 - 0.55}{0.45} \right) \times (-1.732) + (X1 - 2) \times \left( \frac{X2 - 10}{5} \right) \times \left( \frac{X3 - 0.55}{0.45} \right) \times (4.437)
+ (X1 - 2) \times \left( \frac{X1 - 2}{2.23} \right) + (X2 - 10) \times \left( \frac{X2 - 10}{5} \right) \times (-1.751)
\]

\[(1)\]

Figure 1. Plot of the predicted and actual data.

Figure 2. Profile plot of influence in every factors on \( Y_1 \) of DPPH effect.

To convince the result of the influences from the factors to \( Y_1 \), it was showed in Table 3 which found the signification of the influence or probably the relation between the factors. In theory, the increasing of t ratio will be followed by the decreasing of p-value [12]. The influence will be more significant to the response if the p-value is getting smaller. According to the results from Table 3, the p-values of solid to liquid ratio (\( X_2 \)) is less than confidence level at 0.05, which indicated that the influence of \( X_2 \) factors is significant to the response \( Y_1 \). The relation between factors can be identified and it showed that there is significant influence in \( X_2X_3 \)(between solid to liquid ratio and concentration of enzyme).

| Term          | Estimate | Standard Error | t ratio | p-value   |
|---------------|----------|----------------|---------|-----------|
| Intercept     | 81.89    | 1.422          | 57.57   | <.0001*   |
| \( X_1 \)     | 0.670    | 0.871          | 0.76    | 0.4830    |
| \( X_2 \)     | 7.207    | 0.871          | 8.27    | 0.0004*   |
| \( X_3 \)     | 0.487    | 0.871          | 0.56    | 0.6002    |
| \( X_1X_2 \)  | -1.945   | 1.232          | -1.55   | 0.1809    |
| \( X_1X_3 \)  | -1.731   | 1.232          | -1.41   | 0.2188    |
| \( X_2X_3 \)  | 4.437    | 1.232          | 3.60    | 0.0155*   |

Table 3. Parameter influence of factors on \( Y_1 \)
Moreover, Figure 2 showed the influence of every factors on the $Y_1$ response. The DPPH effect was attained at $X_2$ and it is gradually increase, nevertheless, the minimum percentage of free radical scavenging activity of $X_1$ at 2 hours, and at $X_3$ is not significant factor for response $Y_1$.

3.2. Reducing power (Abs)

From Table 2 of box behnken design which also set 15 experimental runs for the reducing power ($Y_2$), it showed that the 7th run achieved maximum result which was attained at absorbance (Abs) 0.473 in the operating factors of hydrolysis time ($X_1$) at 3 hours, solid to liquid ratio ($X_2$) at 1:10, and enzyme concentration ($X_3$) at 0.1 %.

Based on the 15 experimental runs with the factors of reducing power ($Y_2$), a second-order polynomial equation was obtained by the regression and it expressed in this following equation (2).

\[
Y_2 = 0.44 + 0.006 \times (X_1 - 2) + (0.076) \times \left(\frac{X_2 - 10}{5}\right) + (-0.025) \times \left(\frac{X_3 - 0.55}{0.45}\right) + (X_1 - 2) \times \left[\frac{X_2 - 10}{5}\right] \times (-0.016) + (X_1 - 2) \times \left[\frac{X_3 - 0.55}{0.45}\right] \times (-0.010) + (X_2 - 10) \times \left[\frac{X_3 - 0.55}{0.45}\right] \times (-0.011) + (X_1 - 2) \times (X_2 - 10) \times (-0.015) + (X_2 - 10) \times \left[\frac{X_3 - 0.55}{0.45}\right] \times (-0.017) + (X_3 - 0.55) \times \left[\frac{X_2 - 10}{5}\right] \times (-0.013)
\]

(2)

The plot taken from the result of experiment of the reducing power and the prediction result from Equation 2 shows in Figure 3. Based on the plot, it indicated that almost all of predictions data are fit with the result data from the experiment. Moreover, it was convinced by $R^2$ value that reached 0.96.

Figure 3. Plot of the predicted and actual data.  

Figure 4. Profile plot of influence in every factors on $Y_2$ of the reducing power.

The same like the previous result about the influences of the factors on $Y_1$, for the response $Y_2$ was also showed in Table 4 which pinpoint the signification of the influence or probably the relation between the variables. The influence tends to be more significant to the response if the p-value is getting smaller, but it is vice versa with the t ratio which is bigger. According to the results from Table 4, the p-values of
X₂ also is less than confidence level at 0.05, which indicated that the influence of X₂ factor is significant. However, there are not found any significant from the relation between the variables.

\[ Y_2 = b_0 + b_1 X_1 + b_2 X_2 + b_3 X_3 + b_{12} X_1 X_2 + b_{13} X_1 X_3 + b_{23} X_2 X_3 \]

*Table 4. Parameter influence of factors on Y₂*

| Term       | Estimate | Standard Error | t Ratio | p-value |
|------------|----------|----------------|---------|---------|
| Intercept  | 0.44     | 0.016          | 27.20   | <.0001* |
| X₁         | 0.005    | 0.010          | 0.47    | 0.6576  |
| X₂         | 0.078    | 0.010          | 7.90    | 0.0005* |
| X₃         | -0.025   | 0.010          | -2.47   | 0.0501  |
| X₁X₂       | -0.014   | 0.014          | -0.99   | 0.3688  |
| X₁X₃       | -0.014   | 0.014          | -0.99   | 0.3688  |
| X₂X₃       | -0.011   | 0.014          | -0.77   | 0.4776  |
| X₁X₁       | -0.015   | 0.015          | -1.00   | 0.3644  |
| X₂X₂       | -0.117   | 0.015          | -8.03   | 0.0005* |
| X₃X₃       | -0.013   | 0.015          | -0.88   | 0.4206  |

*Significant

Figure 4 showed the profile of the influence of every factor on Y₂. The highest reducing power was achieved at X₂ at 1:12, however, the influence of X₃ is gradually decrease while X₁ is not significant factor for response Y₂.

3.3. Ferrous ion chelating ability

From Table 2 of box behnken design which also set 15 experimental runs for the ferrous ion chelating ability (Y₃), it showed that the 1ˢᵗ run achieved maximum result which was obtained at 49.63% in the operating factors of hydrolysis time (X₁) at 1 hours, solid to liquid ratio (X₂) at 1:10, and enzyme concentration (X₃) at 1 %.

Based on the 15 experimental runs with the factors of ferrous ion chelating ability (Y₃), a second-order polynomial equation was obtained by the regression and it expressed in this following equation (3).

\[ Y₃ = 40.56 + (-1.390) × (X₁ - 2) + (2.544) × \left( \frac{X₂ - 10}{5} \right) + (1.012) × \left( \frac{X₃ - 0.55}{0.45} \right) + (X₁ - 2) × \left( \frac{X₂ - 10}{5} \right) × (X₁ - 2) \times (X₃ - 0.55) × (0.45) \]

\[ + \left( \frac{X₂ - 10}{5} \right) × \left( \frac{X₃ - 0.55}{0.45} \right) × (-0.799) + (X₁ - 2) × [(X₂ - 2) × (2.511)] + \left( \frac{X₂ - 10}{5} \right) × \left( \frac{X₃ - 0.55}{0.45} \right) × (0.681) + \left( \frac{X₃ - 0.55}{0.45} \right) × (4.195) \]

\( Y₃ = 40.56 + (-1.390) × (X₁ - 2) + (2.544) × \left( \frac{X₂ - 10}{5} \right) + (1.012) × \left( \frac{X₃ - 0.55}{0.45} \right) + (X₁ - 2) × \left( \frac{X₂ - 10}{5} \right) × (X₁ - 2) \times (X₃ - 0.55) × (0.45) \]

The plot which was drawn between the actual results from experiment of reducing power and the predictions results from Equation 3 shows in Figure 5. Based on the plot, it indicated that almost all of predictions data are fit with the result data from experiment. Moreover, it was convinced by R² value that reached 0.95 which is the same like the Y₁.
Figure 5. Plot of the predicted and actual data.

Figure 6. Profile plot of influence in every factors on $Y_3$ of the ferrous ion chelating ability.

The influences of variables on $Y_3$ was showed in Table 5 which found the signification of the influence or probably the relation between the factors. The influence tends to be more significant to the response if the p-value is getting smaller, but vice versa with the t ratio which is bigger. According to the results from Table 5, the p-values of hydrolysis time ($X_1$) and solid to liquid ratio ($X_2$) are less than confidence level at 0.05, which indicated that the influence of $X_1$ and $X_2$ factors are significant. However, there are not found any significant from the relation between the variables.

Table 5. Parameter influence of factors on the response $Y_3$.

| Term     | Estimate | Standard Error | t Ratio | p-value |
|----------|----------|----------------|---------|---------|
| Intercept| 40.56    | 0.789          | 51.37   | <.0001* |
| $X_1$    | -1.398   | 0.484          | -2.89   | 0.0341* |
| $X_2$    | 2.544    | 0.484          | 5.26    | 0.0033* |
| $X_3$    | 1.012    | 0.484          | 2.09    | 0.0906  |
| $X_1X_2$ | -0.026   | 0.684          | -0.04   | 0.9712  |
| $X_1X_3$ | 0.403    | 0.684          | 0.59    | 0.5810  |
| $X_2X_3$ | -0.799   | 0.684          | -1.17   | 0.2951  |
| $X_1X_1$ | 2.511    | 0.712          | 3.53    | 0.0168* |
| $X_2X_2$ | 0.681    | 0.712          | 0.96    | 0.3825  |
| $X_3X_3$ | 4.195    | 0.712          | 5.89    | 0.0020* |

*Significant

Figure 6 showed the profile of the influence of every factor on $Y_3$. The ferrous ion chelating ability was attained at $X_2$ is gradually increase, however the minimum ferrous ion chelating ability were observed at $X_1$ at 2 hours and $X_3$ at 0.55%.

4. Conclusion
In this study, antioxidant properties of the extract of saponin from Sapindus mukorossi (soapberry) was analyzed and optimized using Response Surface Methodology. The optimum percentage of Y1 was attained in 91.56 % (X1 = 1 hour, X2 = 1:15, X3 = 0.55 (%). The influences of X2 and X3 on the response Y1 were remarkable. The optimum Y2 was attained in 0.473 Abs (X1 = 3 hour, X2 = 1:10, X3 = 0.1 %). The influences of X3 on the response Y2 was remarkable. The optimum percentage of Y3 was attained in 49.63 % (X1 = 1 hour, X2 = 1:10 (w/v), X3 = 1 %). The influence of X1 and X2 on the Y3 were remarkable. The extract of saponins from Sapindus mukorossi shows excellent in antioxidant capacity based on reducing power and DPPH free radical scavenging test, but it is relatively weak in its ferrous ion chelating ability.

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