Optimization infusion condition on flavonoid and antioxidant activity of herb beverages using response surface methodology

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Abstract. Herbs have potential to be developed as functional beverages to reduce the risk of Covid-19 infection. One of herb ready to serve form is brewed in a bag. This study optimizes the conditions for herb beverages infusion in a silk bag based on their flavonoid content and antioxidant activity. Optimization is completed using the Response Surface Methodology with 3\textsuperscript{3} Central Composite Design models. As the factor X1 is the mass of the herbs in the bag, X2 is the infusion frequency and X3 is the steeping time. Each factor consists of 3 levels. The flavonoid content was determined using the UV-Vis spectrophotometric method. The antioxidant activity was measured based on the DPPH free radical scavenging capacity. The results of this study indicate that the optimal condition is achieved in the herb mass of 1.2 g with an infusion frequency of 18 times and steeping time for 15 minutes. Flavonoids content and antioxidant activity in herb beverages at optimal infusion conditions were 942,353 µg QE / mL (117.79 mg QE / g) and 83.06\%, respectively.

1. Introduction
Covid-19 has become a pandemic worldwide according to a statement from WHO [1]. In Indonesia, until 6 August 2020, 118,753 people were confirmed positive for Covid-19. The mortality rate for sufferers reached 4.6\% of confirmed cases [2]. Every day, confirmed cases continue to increase with a significant increase in number. However, until this writing, there has not been found a drug or vaccine that can cure Covid-19.

The mortality rate for Covid-19 is greatly influenced by comorbidities in patients. In Indonesia, one of the comorbidities that many positive COVID-19 patients suffer from is diabetes mellitus (DM) by 36\%. Cases of death in COVID-19 patients who have a history of comorbidities are 16.8\% [3]. These facts indicate that DM can increase the risk of COVID-19 infection and death of positive COVID-19 cases. High blood sugar levels in humans can increase the ACE2 receptor as receptor binding domain for COVID-19 virus infection into the cells and organs of the human body. In addition, high blood sugar levels in DM sufferers reduce immunity so that they are easily infected with the COVID-19 virus. Furthermore, the condition of high blood sugar in humans causes the body to produce immune blocking genes such as IL-6 and TNF-\textalpha which can trigger cytokine storms, thereby increasing the severity of the condition and the risk of death for COVID-19 positive patients. [4].

Due to no vaccine or specific drug has been found now days that can cure positive cases of COVID-19, so that people are very vulnerable to infection with the COVID-19 virus. One way to reduce the risk of COVID-19 infection is to control blood sugar and cholesterol levels for both people with DM and
non-DM [5]. Indonesian people use herb plants to help reduce the risk of impact or prevent Covid-19 infection.

Our previous studies showed that the combination of several herb plants and named Steamori can make the fasting blood sugar levels of streptozotocin (STZ) induced rats from DM to normal in just 28 days of treatment. Furthermore, this herb solution can increase the amount and basal blood insulin sensitivity of DM rats accompanied by low cholesterol levels with better results than the positive control for the drug metformin. These results indicate that the STeaMori herbs combination has great potential to control blood sugar and cholesterol so that it can reduce the risk of COVID-19 infection. Steamori herbs contain flavonoid compounds and antioxidant activity that have health effects on the body [6].

Herb bag is one form of practical packaging for consuming herbal beverages. Bioactivity and health benefit are affected by bioactive compounds contained in herb beverages. The optimal condition of herbal beverages infusion needs to be studied so that herbal beverages contain optimal bioactive flavonoid and antioxidant activity when consumed. The serving conditions that can be optimized include herb mass, infusion frequency and steeping time of herb bag in hot water. The purpose of this study is to optimize infusion condition of herb bag Steamori based on flavonoid and antioxidant activity.

2. Materials and method

2.1. Materials
Steamori herbal samples powder with particle size of 100 mesh were obtained from the herbal industry of PT. Sinar Herba Radix in Tangerang, Indonesia. Moisture content of herbal samples is less than 10%. Quercetin standard with purity > 99% based on HPLC was obtained from Sigma Aldrich (USA). The DPPH, NaNO$_2$ and Al (NO$_3$)$_3$ reagents were obtained from E-Merck, Germany.

2.2. Method

2.2.1. Optimization of Herb ready to serve infusion conditions
Optimization of infusion conditions for the Steamori herb bag comprise mass of steamori herbs packed in silk bags (0.19 – 2.21 g). Infusion was held by immersed all bag parts into hot water and repeat immersed after previous treatment. Infusion frequencies applied were 2 - 22 times. Steeping treatment was incubating herb bag in hot water used for certain period. Steeping time optimized were 0 – 31.82 minutes. Herb bag is brewed using 150 mL hot water at 75°C. Optimization was carried out based on the flavonoid content and antioxidant activity of the drink.

2.2.2. Determination of flavonoid
A total of 2.0 mL of sample was added with 0.2 mL of NaNO$_2$ 5% then allowed to stand for 6 minutes. The solution was added 0.2 mL of Al (NO$_3$)$_3$ 10% and let stand for 6 minutes. Subsequently, the solution was added with 2 mL of NaOH 4% and let stand 15 minutes. The absorbance of the solution is measured using a UV-Vis spectrometer (Shimadzu 1240) at a wavelength of 501 nm ($A_{501}$). Total flavonoid content were measured based on the standard curve between the standard curve of quercetin vs $A_{501}$. Total flavonoid are expressed as µg quercetin equivalent / mL sample (µg QE / mL sample) [7].

2.2.3. Antioxidant activity
The antioxidant activity was determined based on the DPPH free radical scavenging capacity based on the method [8]. A total of 0.25 mL of sample was added with 1.0 mL of 0.4 mM DPPH solution in ethanol. The solution was homogenized with vortex and incubated for 30 minutes. The solution was then measured for its absorbance at a wavelength of 515 nm ($A_{515}$). The blank is ethanol solution while the control solution is 0.4 mM DPPH solution. Antioxidant activity is expressed as the percentage of DPPH inhibition according to equation (1):

$$\text{Inhibition DPPH} \%(\%) = \frac{(A_{515 \, \text{control}} - A_{515 \, \text{sample}})}{A_{515 \, \text{control}}} \times 100\% \quad (1)$$
2.2.4. Data analysis
Optimization was carried out by using the Response Surface Methodology (RSM) design using the $3^3$ Central Composite Design (CCD) model. As factor X1 is the mass of Steamori herbs (0.6; 1.2; 1.8 g), factor X2 is the frequency of infusion (6; 12; 18 times) and factor X3 is the steeping time (5; 15; 25 minutes). Prediction model and lack of fit test used ANOVA with $P$ value = 0.05. The prediction model obtained is used to determine the optimal conditions for infusion condition of herbs bags based on total flavonoid content and antioxidant activity.

3. Result and discussion
Optimization of Steamori herb infusion conditions is carried out to obtain optimal flavonoid content and antioxidant activity. Factors that can influence the flavonoid and antioxidant activity in herb infusion are herb mass/water ratio and steeping time [9]. Bioactive compound contained in herb is flavonoids. In the herb plant matrix system, the bioactive compounds of herb can provide bioactivity both individually and in combination [10]. It is necessary to optimize the condition for herb infusion condition. The results of total flavonoids and antioxidant activity in the optimization design for Steamori herb infusion conditions are shown in table 1.

Table 1. Total flavonoids and antioxidant activity on the optimization of the Steamori herb infusion conditions design

| Run | X1, mass (g) | X2, steeping frequent (time) | steeping time (minutes) | Flavonoid (µg QE/mL) | Antioxidant Activity (% inhibition) |
|-----|--------------|-----------------------------|-------------------------|----------------------|------------------------------------|
| 1   | 0.19         | 12                          | 15                      | 193.33               | 35.98                              |
| 2   | 1.2          | 12                          | 15                      | 809.20               | 86.27                              |
| 3   | 0.6          | 6                           | 5                       | 265.88               | 65.40                              |
| 4   | 1.2          | 12                          | 31.82                   | 410.98               | 81.74                              |
| 5   | 1.2          | 12                          | 15                      | 865.88               | 86.27                              |
| 6   | 1.2          | 12                          | 15                      | 863.92               | 86.66                              |
| 7   | 1.2          | 2                           | 15                      | 644.31               | 85.10                              |
| 8   | 1.8          | 6                           | 5                       | 185.49               | 46.69                              |
| 9   | 1.2          | 12                          | 15                      | 763.92               | 82.76                              |
| 10  | 1.2          | 22                          | 15                      | 789.41               | 83.66                              |
| 11  | 1.8          | 6                           | 15                      | 483.53               | 79.43                              |
| 12  | 0.6          | 18                          | 5                       | 465.88               | 85.97                              |
| 13  | 2.21         | 12                          | 15                      | 624.71               | 89.15                              |
| 14  | 1.2          | 12                          | 15                      | 797.26               | 83.98                              |
| 15  | 1.8          | 18                          | 25                      | 489.41               | 83.62                              |
| 16  | 1.8          | 18                          | 5                       | 420.78               | 85.25                              |
| 17  | 1.2          | 12                          | 0                       | 422.75               | 86.85                              |
| 18  | 0.6          | 18                          | 25                      | 483.53               | 80.25                              |
| 19  | 1.2          | 12                          | 15                      | 822.75               | 83.88                              |
| 20  | 0.6          | 6                           | 25                      | 444.31               | 78.86                              |
3.1. Flavonoid
One of the bioactive compounds contained in Steamori herbs are flavonoids. Flavonoids have various bioactivity which are included antioxidant activity. The ability of flavonoid compounds as antioxidants is due to the capacity of flavonoid compounds in donating electrons and hydrogen atoms from their polyhydroxy groups to scavenge free radicals. The capacity of the donor and the hydrogen atom of the flavonoid compound is linearly proportional to the concentration of the flavonoid compound [11]. The optimization of Steamori herbs infusion conditions resulted in a quadratic model equation. The ANOVA assay of model prediction and the lack of fit are shown in table 2.

| Table 2. ANOVA for response surface quadratic model of flavonoid. |
|-----------------|---------|---------|---------|---------|
| Source          | Sum of squares | df      | Mean square | F value | p-value |
| Model           | 7.517E+005    | 9       | 83523.04    | 58.08   | <0.0001 |
| Lack of fit     | 2248.03      | 2       | 1112.02     | 0.72    | 0.5316  |

Based on the ANOVA assay, the prediction model shows a significant model. The lack of fit test shows not significant, which means that the predicted and actual data are not significantly different. Factors that had a significant effect of flavonoid content in beverages were infusion frequency (X2) and steeping time (X3). The R² of the model achieved was 0.9868 and the adjusted R² was 0.9698. The mathematical model for predicting flavonoid content based on infusion conditions is shown in equation (2) as follows:

\[ y = -582.47920X1^2 - 0.90256X2^2 - 1.68593X3^2 - 3.28461X1X2 + 1.54257X1X3 - 0.61176X2X3 + 142.36577X1 + 42.44189X2 + 61.42914X3 - 843.60194 \]

(2)

Based on the mathematical model, it can be seen that the effect of herb mass, infusion frequency, steeping time and the interaction of infusion frequency and steeping time have an effect on increasing flavonoids. The increase in herb mass can increase the flavonoid source of the sample. The frequency of infusion and steeping time have an effect on the longer contact time of the sample as a source of flavonoids with hot water as a solvent so that the solubility of flavonoids in the solvent increases. Hot water and the processing of herbal plants into powder can cause cells to break easily and metabolite compounds to diffuse into the solvent. The diffusion of the active compound can also increase with the longer contact time between herb cells and hot water. The polarity of a compound greatly affects its solubility in a solvent. The use of hot water with a temperature of 75–80°C is due to increase the solubility of flavonoid compounds. However, the use of temperatures above 50°C needs to be limited in steeping time because longer steeping time of herb in hot water will degrade flavonoid compounds. [12]. The square of steeping time actually has the effect of lowering flavonoid levels. Furthermore, it is necessary to optimize the steeping time which provides higher levels of flavonoids. Surface plot of model prediction is displayed as shown in figure 1.

3.2. Antioxidant activity
The antioxidant activity was determined based on the inhibition of DPPH free radicals by electrons from the active compounds contained in herb beverages. The higher the electron and hydrogen donor capacity by the active compound, the higher the antioxidant activity [11]. One of bioactive compounds that have antioxidant activity is the flavonoid group [10]. Based on the ANOVA assay, the prediction model obtained was significant while the lack of fit test gave not significant results (table 3).

R² value of model prediction is 0.9888 and the adjusted R² is 0.9719. The ANOVA assay showed that there was a significant interaction between the mass of herbs (X1) and steeping time (X3). Interaction also occurred between the frequencies (X2) and steeping time (X3). The square of each factor also has a significant effect. The mass factor (X1) and infusion frequency (X2) independently had a
significant effect, but the steeping time factor (X3) independently had no significant effect on antioxidant activity. The prediction model equation achieved is shown in equation (3).

\[ y = 11.62542X_1^2 - 0.22668X_2^2 - 0.001086X_3^2 + 0.15381X_1X_2 + 0.82771X_1X_3 \\
- 0.20204X_2X_3 - 49.04170X_1 + 10.51477X_2 + 1.36732X_3 + 32.51162 \]  

(3)

**Figure 1.** Surface plot of flavonoid prediction quadratic model

**Table 3.** ANOVA for response surface quadratic model of antioxidant activity.

| Source   | Sum of squares | df | Mean square | F value | p-value Prob > F |
|----------|----------------|----|-------------|---------|-----------------|
| Model    | 1625.73        | 9  | 180.64      | 58.68   | < 0.0001        | significant |
| Lack of fit | 5.18     | 1  | 5.18        | 1.95    | 0.2214          | not significant |

The mass herb as independent factor (X1) had the highest effect on lowering antioxidant activity. High mass of herb will lower the mass to solvent ratio which can reduce the extraction capacity of the solvent to dissolve the active compound. In addition, the higher the mass of herb extracted, the lower the antioxidant activity because it is possible for many non-antioxidant compounds to be dissolved in the beverage matrix. The interaction between mass and infusion frequency (X1.X2) increased the antioxidant activity but the increase did not have a significant effect. In this study, one of the critical conditions is the interaction between mass and steeping time (X1.X3) which has a significant effect on increasing antioxidant activity. The mass ratio to the solvent, temperature and contact time can affect the quantity of dissolved bioactive compounds [13] so that it will affect its antioxidant activity. In this study, it was found that mass factor independently actually reduced antioxidant activity, but squared and its interactions with other factors could increase antioxidant activity. Therefore, it is necessary to optimize the mass of herbs and other factors to optimize the dissolving capacity of compounds that have antioxidant activity. Figure 2 shows the surface plot of the prediction model for antioxidant activity.

A correlation assay was also carried out between the total of flavonoid compounds and antioxidant activity. The results of the correlation test gave a correlation value of 0.76. Correlation indicates that the content of flavonoid compounds with antioxidant activity has a positive correlation. Total flavonoid content is linear proportionally affect antioxidant activity of beverages. However, it needs to be investigated more deeply whether the quality or type of flavonoid compounds or the quantity of flavonoid compounds is more affected to antioxidant activity.
3.3. Optimization infusion condition
Optimization of infusion conditions is carried out using targeted optimization. The conditions chosen are those that give desirability > 0.90; flavonoids > 800 µg QE / mL and antioxidant activity > 80%. Based on the optimization, the optimum conditions achieved were 1.2 g of herb mass with an infusion frequency of 18 times and a steeping time of 15 minutes. Total Flavonoid content and antioxidant activity of herb beverages at optimum infusion conditions were 942.353 µg QE / mL (117.79 mg QE / g) and 83.06%, respectively.

4. Conclusion
The optimum infusion condition for Steamori herb beverages based on total flavonoid content and antioxidant activity is a herb mass of 1.2 g with an infusion frequency of 18 times and a steeping time of 15 minutes. The critical factors for optimization were found in the interaction of herb mass and steeping time.

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