

Optimization of protein and tannin extraction in *Moringa oleifera* leaf as antioxidant source

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Article history:

Received: 23 June 2020

Received in revised form: 16 July 2020

Accepted: 9 August 2020

Available Online: 3 October 2020

Keywords:

Moringa leaf,

Extraction optimization,

Total protein,

Total tannin,

Antioxidant activity

DOI:

[https://doi.org/10.26656/fr.2017.4\(6\).293](https://doi.org/10.26656/fr.2017.4(6).293)

Abstract

Moringa oleifera contains high nutritional and bioactive compounds that have the potential as a source of antioxidants. The main objective of this study was to obtain optimal conditions in the extraction process of Moringa leaf, maintain protein and antioxidant activity, and reduce tannin. It is carried out through the response surface method which consists of two factors. The first factor (X_1) was the extraction temperature ($^{\circ}\text{C}$) which contains three levels, namely 70, 80, and 90°C . The second factor (X_2) was time (mins) consisting of three levels, namely 5, 10, and 15 mins. The parameters in this study were total protein, antioxidant activity, and total tannin. The results revealed that the optimum condition for Moringa leaf extraction is found to be temperature 80.54°C and a time of 12.19 mins. In that treatment, the total protein is 17.4594%, antioxidant activity is up to 10.2629 $\mu\text{g/mL}$, and tannin is 7.853% with the desirability of 0.792 or 79.2%.

1. Introduction

Moringa oleifera is a well famous plant cultivated in hot up to tropical climates, such as Indonesia (Mendieta *et al.*, 2013). Organic moringa cultivation in Indonesia has been carried out in several regions such as Blora, Central Java, Gunung Kidul, Yogyakarta, Poteran Island, Sumenep Regency, and NTT Province. One of the parts of the moringa plant that can be utilized is the leaf, where the production of wet leaves is around 5-6 tons/hectare (Krisnandi, 2015).

Moringa leaf contains fiber, protein, fat, minerals such as Ca, Mg, P, K, Cu, Fe, S, Vitamins: A (Beta-carotene), B-choline, B1-thiamine, riboflavin, nicotinic acid, ascorbic acid, acid Amino: Arginine, Lysin, Tryptophan, Phenylalanine, Threonine, Leucine, Methionine, Isoleucine, Valine, Phytochemicals: tannin, sterol, saponin, terpenoid, phenolic, alkaloid, flavonoid, quercetin, isoleucine, valine, phytochemicals: tannins, sterols, saponins, terpenoids, phenolics, alkaloids, flavonoids, quercetin, isoleucine, violins, phytochemicals: tannins, sterols, saponins, terpenoids, phenolics, alkaloids, flavonoids, quercetin, isocercitins, caemferothosrays, glycothiocytes, glycotosicans (Gopalakrishnan *et al.*, 2016). Besides, the iron content in moringa leaf is also higher than other vegetables,

which is 17.2 mg/100 g (Yameogo *et al.*, 2011).

In general, all parts in the moringa plant have several functions, namely as hypolipidemic, antiatherosclerotic, prevention of cardiovascular disease (Chumark *et al.*, 2008), anti-diabetic, inflammation, anemia, and immune system booster because moringa is rich in nutrients, essential amino acids, vitamins, minerals, and 40 natural antioxidants sources (Mahmood *et al.*, 2010).

Nowadays, most research on the use of moringa leaf tend to be directed to the use of natural antioxidants. The antioxidant activity of moringa leaf extract, both young and old, is very effective to prevent oxidative damage of the main biomolecules (Shahriar *et al.*, 2012). This is because the phenol content in a leaf is much higher compared to pods and seeds, so the use of moringa leaf as a source of natural antioxidants is highly recommended (Wangcharoen *et al.*, 2011). Apart from being a source of antioxidant, the leaf also has a function as anti-inflammatory, anti-pain (Upadhye *et al.*, 2011), anti-ulcer (Das *et al.*, 2011), and as an antimicrobial and wound healing (Moyo *et al.*, 2012). In addition to its advantages, moringa has weaknesses, including having a low protein digestibility which is $56.1 \pm 8.9\%$ and there are anti-nutritional substances namely tannins, saponins, and trypsin inhibitors. Production protein concentrates is

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one way to increase digestibility and reduce anti-nutrient properties in moringa leaf (Kholis and Hadi, 2010).

The anti-nutritional substances in moringa leaf can be reduced by heating the leaves in water (Ogbe and Affiku, 2011). Heat extraction treatment is one of the methods to produce protein concentrate from a leaf to create higher protein content compared to the raw materials (Purwitasari *et al.*, 2014).

Taking into account the composition of chemical nutrients, the content of bioactive compounds, antioxidant compounds, and the presence of anti-nutrient substances in moringa leaf, it is necessary to conduct a research on how to use moringa leaf as a source of nutrition, especially as a protein source and natural antioxidants. Furthermore, in order not to damage the protein and bioactive sources of the antioxidants and reduce the tannin content, a simple method should be employed. The purpose of this study was to optimize the extraction conditions of moringa leaf to obtain a high protein with high antioxidant capacity and low tannin extraction.

2. Materials and methods

2.1 Materials

Materials used were fresh *Moringa oleifera* leaves obtained in Sekarmojo Village, Purwosari District, Pasuruan Regency, water as solvent, DPPH (1,1-diphenyl-2-picrylhydrazyl), methanol, ascorbic acid (Rheidel-Hein), sulfuric acid (H₂SO₄), potassium sulfate (K₂SO₄), mercury oxide (HgO), sodium hydroxide-sodium thiosulfate (NaOH-Na₂S₂O₃), saturated boric acid (H₃BO₃), hydrochloric acid (HCl) 0.02 N, red metal indicator, and methyl blue indicator.

2.2 Moringa leaf extraction

Moringa leaf extraction employs the decoction method with a water solvent. The objective of this extraction is to coagulate proteins so that they are easily isolated from non-protein components. Moringa leaves were sorted, weighed and washed. A total of 100 g of wet Moringa leaves were mashed using a blender and added with 100 mL of water. The mixture was filtered using a double filter cloth to separate the fibers from the leaf pulp. The extraction process uses temperature and time based on the treatment. The mixture was then centrifuged for 20 mins to separate the supernatant and sediment. The supernatant was discarded, and the sediment was stored in the refrigerator for further analysis.

2.3 Protein content analysis

A standard protein curve was prepared by placing 0 (blank), 0.1; 0.2; 0.4; 0.6; 0.8 and 1 mL of bovine serum albumin (BSA) into test tubes and topped up with water to 4 mL to make the following concentrations of 6.25 µg/mL, 12.5 µg/mL, 25 µg/mL, 37.5 µg/mL, 50 µg/mL and 62.5 µg/mL, respectively. Then, 5.5 mL of lowry reagent B was added into the test tube and homogenized. The mixtures were allowed to stand for 10 – 15 minutes at room temperature. After that, 0.5 lowry reagent A was added into the test tubes and observed for blue color formation after 30 minutes. The solution was transferred into the cuvette and the absorbance was measured using the UV-Vis spectrophotometer.

For protein content analysis of the sample, 50 g of the extract was mixed with 40 mL of distilled water and centrifuge for 10 mins at 11,000 rpm to obtain the protein precipitate. About 4 mL of the protein precipitate was taken and followed the steps for sample preparation aforementioned by replacing the BSA with the sample. The protein content was determined by measuring the absorbance of the sample and compared against the standard curve (Sulistiyani and Nuril, 2017).

2.4 Tannin content analysis

The tannin content analysis was performed following the method of Chanwitheesuk *et al.* (2004) with slight modification. A total of 0.5 g of the extract was mixed with 10 mL of diethyl ether and incubated for 20 hrs. After incubation, the mixture was filtered and the residue was boiled with 100 mL distilled water for 2 hrs. It was then cooled and filtered. The filtrate was added with distilled water to a volume of the extract (~100 mL) and 0.1 mL of the solution was added with 0.1 mL of Folin Ciocalteu reagent. The mixture was vortex and added with 2 mL of Na₂CO₃ and further vortexed to ensure a complete mix. The absorbance of the mixture was read at a wavelength of 760 nm after an incubation of 30 minutes at room temperature. The results were extrapolated from the standard tannic acid curve prepared following the same procedure by replacing the extract with tannic acid. The total content of tannin was expressed in mg tannic acid/kg extract (Rebaya *et al.*, 2014).

2.5 Antioxidant activity analysis

The antioxidant activity test was carried out by sampling as much as 0.2 mL of extract and added with 3.8 mL of 50 µM DPPH solution. Then, it was homogenized and allowed to stand for 30 mins in the dark. The absorbance was measured at the maximum wavelength of DPPH using the UV-Vis

spectrophotometer. The antioxidant activity of the sample by the magnitude of DPPH radical uptake resistance can be determined by calculating the percentage of DPPH absorption inhibition using the formula:

$$\%Inhibition = \frac{Abs. blank - Abs. sample}{Abs. blank} \times 100 \% \tag{1}$$

The antioxidant activity was determined using IC₅₀ values (50% inhibition concentration). IC₅₀ values of each sample concentration was calculated using the linear regression equation formula (Purwanto *et al.*, 2017).

2.6 Statistical analysis

Statistical analysis was performed using MINITAB Release 14 software. Based on the analysis, the appropriate model for extraction optimization testing was obtained using Design expert 7 from graphs observed responses in the form of regression coefficients, 3D response surface plots, and contour plots.

2.7 Optimization of extraction using the Response Surface Method (RSM).

The experimental design to assess the total yield, total protein, antioxidant activity, and the total tannin is the Response Surface Method. The extraction process used two independent variables, namely temperature and extraction time. The composition of the temperature and time variable was designed using a centralized composite design, with the point for the temperature is 80°C, and the point for the time variable was 10 mins. The mathematical equation model used was as follows:

$$Y = \beta_0 + \sum_{i=1}^2 \beta_i X_i + \sum_{i=1}^2 \beta_{ii} X_i^2 + \sum_{i < j=1}^2 \beta_{ij} X_i X_j \tag{2}$$

Where Y is the response (outcome), β₀ is a constant, β_i, β_{ii}, β_{ij} are the coefficients of the independent variable (X), X is the independent variable with no code (for the extraction temperature variable: extraction temperature (X₁) level 70, 80 and 90°C; extraction time (X₂) level 5, 10 and 15 mins and ε is random error. The level of the independent variables (temperature and time) in this study can be seen in Table 1 (Bharathi and Rajendiran 2011).

Table 1. Level of independent variables, code, and optimized values

Independent	Degree	-1	1	- α	+ α
Temperature (X ₁)	Celsius	70	90	65,85	94,14
Time (X ₂)	Minute	5	15	2.93	17.07

3. Results and discussion

Based on the results, the actual values, responses of protein variable, antioxidant activity (IC₅₀), and tannins

are tabulated in Table 2.

Based on Table 2, protein values ranged from 11.12% at 65.86°C with extraction time of 10 mins to 19.6% at 80°C with 17.07 mins of extraction time. The antioxidant activity (IC₅₀) was between 9.23 μg/mL at 80°C with extraction time of 10 mins to 13.32 μg/mL at 90°C with extraction time of 15 mins. While tannin values ranged from 7.13% at 80°C with extraction time of 17.07 mins to 13.71% at 65.86°C with extraction time of 10 mins. The responses of the total protein variable, antioxidant activity (IC₅₀), and total tannin are explained below.

3.1 Total protein

Moringa leaf is one of the parts of moringa plants that have several benefits and usage. This is because moringa leaves are rich in nutrients, including protein, calcium, iron, vitamin A, vitamin B, and vitamin C (Misra *et al.*, 2014). Besides, moringa leaves are a potential source of protein to overcome the problem of malnutrition (Farooq *et al.*, 2012). The results of the analysis using the response surface method (RSM), obtained from the selection of models based on "Sequential Model Sum of Squares", the selected model is a quadratic because it has a value of 0.0144 (<5%). The probability of model error was less than 5%, so it has a significant effect on the total protein response. Likewise, with the Lack of Fit calculation results, the quadratic model was stated as the "suggested" model.

Based on the analysis of R-square/R² (coefficient of determination) on ANOVA, it has a R² value of 0.8169. This reveals that the data is capable to support the model of 81.69% in which consists of extraction temperature and extraction time. The remaining 18.31% is influenced by other factors that are not included in the model such as the type of solvent, the extraction method, plant varieties, and the growing environment of the plant itself and others (Hernández *et al.*, 2009). The effect of temperature and extraction time on total protein are shown in Figures 1a and 1b.

Figure 1 shows the amount of protein due to the effect of variations in temperature and extraction time. The temperature and extraction time variable values are set to the optimum value at running # 13 RSM experimental design. The lines consisting of points on the contour plot graph that show a combination of two components with different amounts that produce the same protein response value (Myers *et al.*, 1995).

Figure 1a and 1b reveal that the x-axis is the extraction temperature factor and the y-axis is the extraction time factor. The line inside the contour plot is

Table 2. Total protein values, antioxidant activity (IC_{50}), and tannin extract of Moringa leaf

Extraction Temperature ($^{\circ}C$)	Extraction Time (min)	Protein (%)	Antioxidant IC_{50} (mg/mL)	Tannin (%)
80	10	17.83	10.03	8.27
70	5	11.32	13.3	12.5
94.14	10	13.35	12.5	11.95
80	10	16.21	10.63	7.22
80	17.07	19.6	10.89	7.13
80	10	16.89	10.33	8.07
70	15	12.98	12.9	8.87
80	10	16.09	10.23	9.06
90	15	15.67	13.32	11.55
90	5	16.08	10.84	11.2
80	10	17.23	9.23	8.09
80	2.93	11.95	12.23	9.21
65.86	10	11.12	13.05	13.71

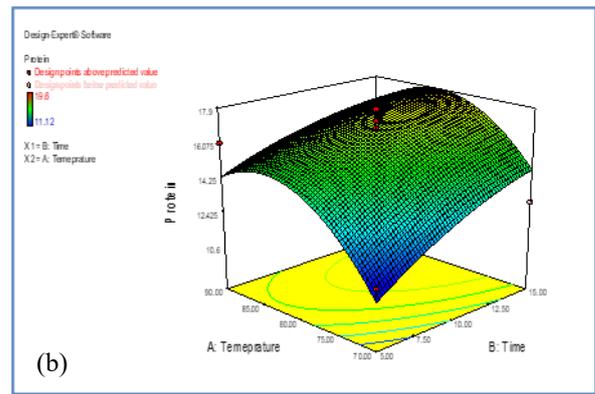
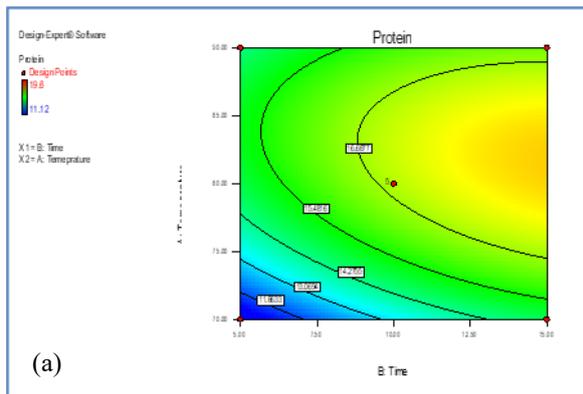


Figure 1. (a). Contour plot and (b) Surface response that shows the effect of temperature and extraction time on the total protein of Moringa leaf extract

the protein response value. Judging by the contour color, the red dot shows the highest protein response value of 19.6%. The blue color indicates the lowest response value of 11.12%. Based on Figure 1, it can be seen that a variation in temperature and extraction time significantly affects the total protein response. The total protein content increased along with the increase of extraction temperature. However, the total protein content decreased after the extraction temperature was above 80°C. The lowest total protein content was at the extraction temperature of 65.85°C at 11.12%. The protein content increased along with the increase of the extraction temperature up to the highest level of 19.6% at 80°C, and then the protein content decreased after the temperature was more than 80°C

Temperature is one of the important factors that play a role in the quality and levels of protein. The increase of extraction temperature can increase the solubility of the protein in the extract. But the higher the temperature, the higher the protein damage which also affects the quality of the protein (Conscience, 2016).

Moreover, the total protein content was observed to be affected by the extraction time and in a proportional sequence. The protein level was higher with a longer extraction time. However, the protein level decreased

after passing an optimum time. Diantika *et al.* (2014) stated that a long extraction time can cause a long heating effect on the material so that the results will increase the saturation point. Jayanudin *et al.* (2014) also added that this heating effect will enlarge the pores of the material so that the solvent can enter through the pores and dissolve the components that are absorbed then, the solute diffuses out the cell wall surface.

Considering trends in the curve, it shows that the higher the extraction temperature and the longer the extraction time, the protein value increases and will decrease again after reaching a certain time and temperature. This is consistent with the opinion of Ahmed (2016) that heating for coagulation of protein concentrates from leafy vegetables is applied at a temperature of 80-90°C. Meanwhile, according to Purwitasari *et al.* (2014), the protein content of the product raises with a long extraction time. And then, it becomes saturated or damaged because more protein is dissolved in the solvent to a certain extent.

3.2 Antioxidant activity (IC_{50})

Antioxidants are bioactive compounds that can inhibit free radicals and substrate oxidation, which have an important role in protecting cells from damage by being able to block the process of oxidative damage

caused by free radicals (Huang *et al.*, 2010). Plants are potent sources of natural antioxidants. Phytochemical compounds found in plants such as flavonoids, phenolic acids, tannins, terpenoids, and alkaloids with a significant antioxidant activity which is very good for maintaining health (Winarsi, 2007).

The model selection results are based on "Sequential Model Sum of Squares", the selected model is quadratic because it has a value of 0.002 (<5%). This shows that the chance of model error is less than 5%, so it has a significant effect on the response of antioxidant activity. Likewise, with the Lack of Fit calculation results, the quadratic model was stated as "suggested", namely the chosen model. While the R-square/R² (coefficient of determination) on ANOVA has a value of 0.8501, this shows the data can support the model of 85.01% in which consists of the treatment of extraction temperature and extraction time. The remaining 14.9% is influenced by other factors that are not included in the model. The effect of temperature and extraction time on the antioxidant activity is presented in Figure 2.

Based on the research results, the extraction temperature has a significant effect on the model. In Figure 2, it can be seen that the antioxidant activity (IC₅₀) will decrease along with the increase of extraction temperature. But the antioxidant activity (IC₅₀) increased at the extraction temperatures above 80°C. Likewise, with the extraction time treatment, the graph clearly shows that antioxidant activity (IC₅₀) will increase after the extraction time of 10 minutes.

In Figure 2, the red color shows the highest antioxidant response value of 13.32. mg/mL. The blue color indicates the lowest response value of 9.23 mg/mL. Considering the trends in the curve, it shows that the higher the temperature and time increase, the antioxidant value decreases and then rises after passing through the critical point. According to Belyagoubi *et al.* (2016) temperature is a very important parameter in the food

industry related to the processing, extraction, and storage of food ingredients. The IC₅₀ antioxidant activity value based on the results of the analysis using DPPH reagents ranged from 9.23 mg/mL to 13.32 mg/mL. This shows that the antioxidant value (IC₅₀) in moringa leaf extract for all treatment variations is in the very strong antioxidant group, <50 mg/mL.

3.3 Total tannin

Tannin is a very complex organic compound and can be found in various plants. Tannins can bind to proteins so that proteins in plants can be resistant to degradation by protease enzymes in plants (Dewi *et al.*, 2019). Tannins are chemical compounds that are classified as polyphenol compounds (Deaville *et al.*, 2010). Desmiaty *et al.* (2008) explain that tannins are very complex organic substances, which are composed of phenolic compounds which are difficult to separate and function as natural antioxidants.

Based on the analysis results, the model selection is based on the "Sequential Model Sum of Squares", the selected model is quadratic because it has a value of 0.0002 (<5%). This shows that the chance of model error is less than 5%, so it has a significant effect on the response of antioxidant activity. Likewise, with the Lack of Fit calculation results, the quadratic model is stated as "suggested", namely the chosen model. While R-square/R² (coefficient of determination) on ANOVA has a value of 0.9256, this shows the data can support a model of 92.56% in which consists of the treatment of extraction temperature and extraction time. The remaining 7.44% is influenced by other factors that are not included in the model. The effect of temperature and extraction time on the total tannin content is presented in Figure 3.

Figure 3 shows that the treatment of temperature variations decreases the tannin extract. However, it is stable and tends to rise again after passing its optimum temperature. While the effect of extraction time decreases the amount of tannin along with the addition of

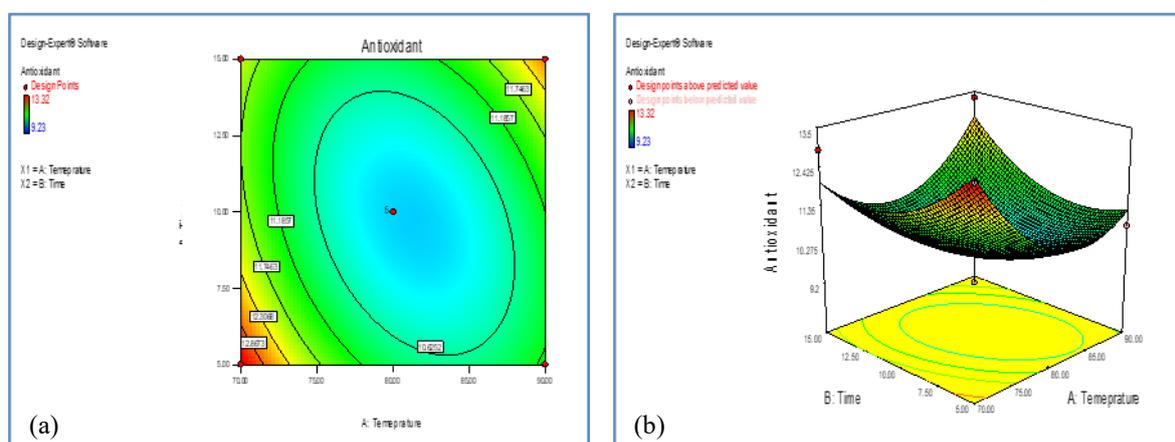


Figure 2. (a). Contour plot and (b) Surface response that shows the effect of temperature and extraction time on the antioxidant activity of Moringa leaf extract

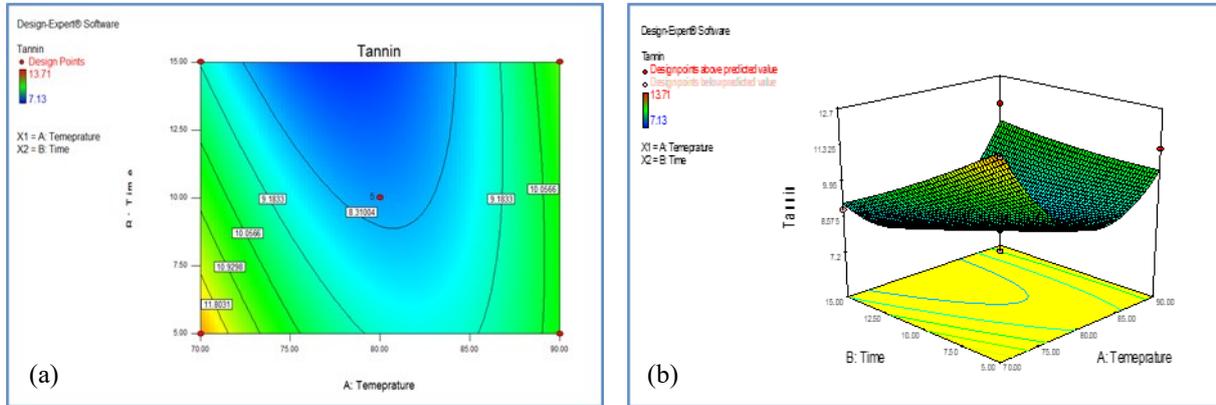


Figure 3. (a) Contour plot, (b) Surface response showing the effect of temperature and extraction time on total tannin in Moringa leaf extract

extraction time. This happens because the longer the extraction process, the tannin compounds contained in moringa leaves undergo an enzymatic oxidation reaction, while the increase in tannin levels occurs due to the inactivation of the catechol oxidase enzyme at higher temperatures (Purnama *et al.*, 2015).

Based on the analysis results, it is reported that the critical value is 80°C and 10 mins. At that point, the total tannin is predicted to reach 8.07%. However, at temperatures above 80°C, the tannin level increased again. This is because the phenol level is disturbed due to the high temperature in the extraction process. In this study, tannin levels will be better when acquiring fewer tannins. This is due to the nature of tannin as an anti-nutrient compound.

Tannin is known as an anti-nutrient compound because of their ability to form complex bonds with proteins. The ability of tannins to precipitate these proteins because tannin has several functional groups that can form strong complexes with protein molecules, therefore tannin is generally regarded as detrimental anti-nutrients. The bond between tannin and protein is so strong that protein cannot be digested by the digestive tract (Matteo *et al.*, 2010).

3.4 Response optimization

Based on the calculation, optimization results using

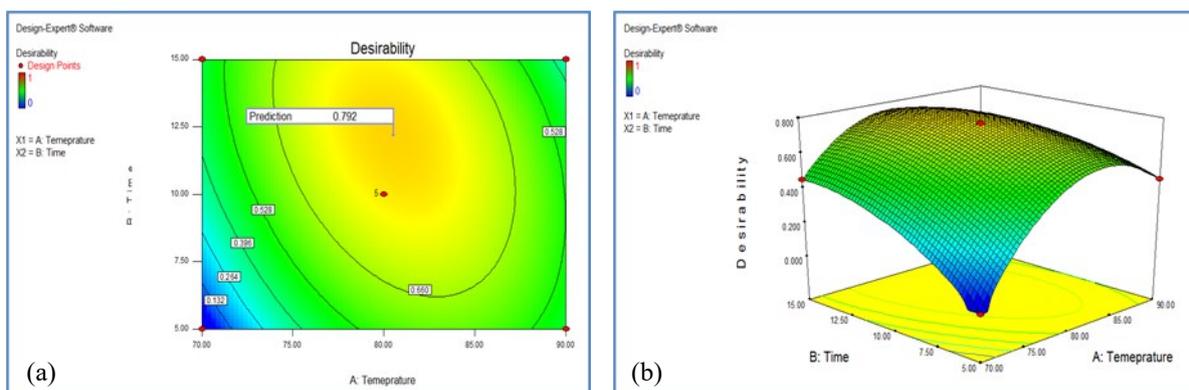


Figure 3. (a) Contour plot, (b) Surface response showing the effect of temperature and extraction time on total tannin in Moringa leaf extract

Design Expert Ver. 9 Trial show that the optimal conditions of the moringa leaf extraction process are the extraction temperature of 80.54°C with extraction time of 12.19 minutes. The treatment resulted in a prediction of a total protein response of 17.4594%, and antioxidant activity of 10.2629 mg/mL, and tannin 7,853 with the desirability of 0.792 or 79.2%. The contour plots and response surface of the predicted results are presented in Figure 4.

According to Bezerra *et al.* (2008), desirability shows the desired scale for each response and determines the accuracy degree of the optimal solution. The desirability value in this study is 0.792 or 79.2%, where the value is getting closer to the value of 1, the accuracy is higher. The scale range for desirability value is 0 to 1, where a value of 0 indicates an unwanted response, while a value of 1 indicates a fully desirable response.

The results of the research on total protein and tannin parameters have the opposite graph trend, where the higher temperature treatment and the longer extraction time will increase the amount of protein, but the amount of protein will decrease again along with the increase in temperature and extraction time. This is contrary to the amount of tannin where tannin will decrease along with the increase in temperature and extraction time, but the tannin value will rise again after passing the optimum point. This is consistent with the statement of Purwitasari

et al. (2004) that the protein content of the product rises with longer extraction time, and then it will decrease to a certain extent because more protein is dissolved in the solvent and becomes saturated or damaged. Although the protein and tannin parameters show the opposite results, the two have a close relationship. As Makkar (1993) stated that the presence of several functional groups in tannins will cause protein deposition when the amount of tannin increases and vice versa. Besides, tannins also bind to mucosal proteins that they affect the absorption of nutrients. In general, tannins are considered as harmful anti-nutrients. Besides, the presence of tannin in moringa leaf extracts must also be minimized, this is to reduce the taste of bit or chelate in the final product.

The effect of temperature and extraction time does not only occur on the amount of protein and tannin, but also the antioxidant activity. Antioxidant activity will decrease when the extraction temperature is above 80°C and the extraction time is more than 10 mins. Prolonged extraction time and higher temperatures will cause oxidation of bioactive compounds and antioxidant activity (Chew et al., 2011). Based on the results of the study, the decoction extraction technique using water as the solvent has a significant effect on the amount of tannin, protein, and the antioxidant activity. Besides, the extraction method using water as the solvent has the prospect of being developed further on a larger scale, because the technique is simple, safe, and easy to apply when compared to the other methods (Vongsak et al., 2013).

4. Conclusion

RSM is an appropriate and effective method for optimizing the extraction conditions of moringa leaves using a water solvent. The optimum condition for maintaining the amount of protein and antioxidant activity and reducing tannin content in moringa leaf extract is at an extraction temperature of 80.54°C with an extraction time of 12.19 mins. It can be predicted that the extract will contain 17.4594% of total protein, 10.2629 µg/mL of antioxidant activity and 7, 853 tannin.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgement

Thank you to the promoters and co-promoters, all lecturers, staff, and laboratory staff at the Faculty of Agricultural Technology, Universitas Brawijaya and Faculty of Agriculture, Yudharta University who have helped in the research and preparation of this scientific article. Also, thank you to the LPDP for funding this

research.

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