Hue University Journal of Science: Natural Science
Vol. 133, No. 1B, 35–48, 2024

pISSN 1859-1388
eISSN 2615-9678

Optimizing conditions to improve polyphenol content and screening antioxidant capacity with DNA protection activity of *Perilla frutescens*

Thien-Kim Nguyen Ngoc [1,2], Anh-Vu Truong Huynh[3], Khue-Tu Nguyen Hoang[1,2]*

[1] School of Biotechnology, HCMC International University, Vietnam
[2] Vietnam National University HCMC, Vietnam
[3] Microbiology laboratory, Center of Analytical Services and Experimentation HCMC (CASE), Vietnam

* Correspondence to Khue-Tu Nguyen Hoang <nhktu@hcmiu.edu.vn>

(*Received: 31 January 2023; Revised: 29 August 2023; Accepted: 25 October 2023*)

Abstract. Polyphenols are among the natural antioxidants that have been exploited in recent years for their safe and effective ability against oxidative stress. This project aimed to optimize 3 factor-conditions affecting total polyphenols extracted from *Perilla frutescens* (L.) Britt as well as DNA protection activity of polyphenols for applications in the fields of dietary supplements, pharmaceuticals, and health care. All extracts contained phenolic compounds and exhibited good antioxidant ability through a ferric reducing antioxidant power assay. The total polyphenolic compounds varied from 6.056 ± 0.08 to 9.630 ± 0.127 mg of gallic acid equivalents (GAE) per 1 g dry weight (dw). The highest phenolic content yield was extracted at 70°C for 60 minutes at a pH of 7.0. However, a sample with the highest polyphenol content had a lower residual DNA concentration than the extract (55°C, 60 min, and pH 6.0) with the greatest reducing power. The result of the DNA protection assay also indicated that the extraction concentration and pH condition had a significant effect on preventing DNA from being damaged by free radicals. The study found the conditions for improving polyphenol in the extraction of *P. frutescens* (L.) Britt with the aid of Box-Behnken Design. This research also proposed that *P. frutescens* (L.) Britt is a good source showing DNA protection and antioxidant activity for healthcare.

Keywords: *Perilla frutescens* (L.) Britt, optimization, Box-Behnken design (BBD), total polyphenol content (TPC), ferric reducing antioxidant power (FRAP), and DNA protection

1 Introduction

Oxidative stress is an imbalance between the generation of reactive oxygen species (ROS) and the antioxidant defense mechanism (inhibition of free radical production, direct metal chelating and free radical scavenging, and detoxification) [1]. ROS can be formed when oxygen reacts with certain compounds to produce highly reactive and toxic species, such as superoxide anion radical (O^{2-}), singlet oxygen (O_2), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH\bullet$) [2]. The accumulation of ROS in the body results in oxidative stress that can seriously damage cell membranes and other structures such as proteins, lipids, lipoproteins, and deoxyribonucleic acid [3]. Therefore, antioxidant sources are essential for systems to fight against oxidative stress and to contribute to disease prevention that must be continuously restored in the organisms [4].

There are two major sources of antioxidants: metabolic antioxidants, which are endogenous antioxidants created by the body's metabolisms, and nutritional antioxidants, which are exogenous antioxidants obtained from ingestible foods or supplements [5, 6]. However, because humans constantly expose to a variety of

external factors that enhance ROS formation, endogenous antioxidants are insufficient to prevent DNA from being oxidized. Exogenous antioxidants have gained popularity due to their chelating action against free radicals and their ability to reduce the risk of a variety of diseases and cancer in humans [7]. Recently, synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are concerned to have potential adverse effects on people's health [8, 9]. Whereas due to their safety and cost-saving, natural and herbal-derived antioxidants have garnered considerable interest [10]. According to Pizzino, natural antioxidants are promising because of their high effectiveness in terms of ceasing or decelerating the rate of degenerative reactions in the body without side effects [11].

P. frutescens (L.) Britt. belonging to Lamiaceae is a common herbal plant in Asia, including China, Korea, Japan, and Vietnam [12]. *Perilla* is currently gaining public interest due to its natural bioactivities for application in cosmetic formulations [13]. It was found that the total polyphenol content of green leaf chemotypes was relatively lower than the red one, which in turn influenced its antioxidant effects [14]. Consequently, different phytochemotypes can be identified in various *Perilla* cultivars, and they may have differences in the effectiveness of the bioactivities [13]. Vietnamese *P. frutescens* (L.) Britt., whose stem is colored both green and purple, was collected for further research of potential bioactivities.

Box-Behnken Design - Response Surface Methodology (RSM) is used because the number of experimental runs for this design is significantly smaller than other factorial techniques. In Box-Behnken Design (BBD), a model between total polyphenol contents (TPC), ferric reducing antioxidant power (FRAP), and three levels of independent parameters is built to obtain optimal conditions for high polyphenolics and effective antioxidants and purposed to fit a quadratic model [15]. The influence of several factors on the process is also easily identified by using RSM. Factors including temperature, time, and pH values of conventional extraction on polyphenol yield and their effects were studied, followed by the interactions between them. In this study, methanol and other solvents were considered as harmful substances to the environment to prepare sample extracts so the production cost would increase for waste treatment. Accordingly, water was proposed as a low-cost and non-toxic alternative for solvent extraction.

Many previous papers reported on the relationship between total phenolic content, antioxidant capacity, and metal-reducing potential. The effectiveness of antioxidants is believed that the higher extract concentrations of bioactive components in the extract, the stronger antioxidant action as well as the metal-reducing ability [12]. Otherwise, under certain conditions, there are pro-oxidant properties in some plant-derived polyphenols [16]. There is a relative lack of data on optimizing polyphenol output from *P. frutescens* using traditional approaches. A water-bath heating technique is used in this research to achieve maximal extraction through moderate heating. However, this technique does have a certain drawback is that extraction temperatures over 70°C cause rapid polyphenol degradation and reduce the yield [17]. According to Maisuthisakul et al. , polyphenols are pH and temperature sensitive, which can impact the antioxidant efficacy of the plant extract [18]. This is also demonstrated by Zimeri et al. and Zeng et al., who found that extracting and storing polyphenols at low temperatures and acidic pH conditions had no significant effect on polyphenol characteristics. [19, 20]. Therefore, the pH of water

pISSN 1859-1388
eISSN 2615-9678

and temperature as essential factors in the decomposition of polyphenol compounds, and the stability of the antioxidant activity during the extraction was investigated to more appropriate ranges for human body. Moreover, the interaction of extraction time and temperature which determines the extraction rate has a significant impact on polyphenol yields [21].

Therefore, the present work aimed to assess the effects of three condition factors including extraction time, temperature, and pH of solvent on the yield of total polyphenolics via a conventional method. Antioxidant potential in reducing iron radicals was also determined to evaluate correlations between total polyphenolics, antioxidant activity, and free radical-reducing power. Therefore, a further experiment on screening for DNA protection from the oxidative stress process through antioxidant ability from different extracting conditions of *P. frutescens* (L.) Britt extracts can be investigated.

2 Materials and methods

2.1 Materials

Perilla frutescens powder preparation

P. frutescens (L.) Britt was collected randomly from Gia Kiem Commune, Dong Nai Province, and then washed briefly through cold distilled water to remove dust. The plant was dried at 55°C until the consistent weight and ground into powder [22]. The dry powder was stored at room temperature and avoided exposure to light before using for the experiments.

Chemicals

Gallic acid used for standards of both assays was purchased from Sigma-Aldrich. Folin-Ciocalteu (FC) reagent and other chemicals including sodium carbonate (Na_2CO_3), sodium acetate (CH_3COONa), phosphate-buffered saline (PBS), potassium ferricyanide ($K_3Fe(CN)_6$], trichloroacetic acid (TCA), ferric (III) chloride ($FeCl_3$), hydrogen peroxide (H_2O_2), Tris-EDTA (TE) buffer, etc. were used to perform test experiments.

2.2 Methods

Preparation of the extract

Using the water-bath heating method, 2.0 g of *Perilla* powder was extracted with 60 mL distilled water in a chamber at different pH, temperature, and time according to the optimization study design demonstrated in Table 1. After the extraction process, the solutions were cooled at room temperature and filtered with filter paper, then stored at -20°C for future experiments [22].

RSM – Box-Behnken Design of experiments

In this study, three Box-Behnken Design factorials were investigated, including temperature, time, and pH to determine their impact on the extraction process and to identify the conditions with the highest total polyphenol content and the most effective antioxidant potential based on FRAP activity from *P. frutescens.* The FC and FRAP assays were designed with 15 total runs for three independent variables. Each independent variable had three variable levels: low (−1), medium (0), and high levels (+1), and there were also three repeated experiments at a center point (0, 0, 0). The central conditions were repeated to estimate the pure error [22]. In actual experiments, the factors were X_1 - extraction time (60 min, 90 min, 120 min), X_2 - the temperature (40°C, 55°C, and 70°C), and X_3 - pH (pH 6.0, pH 7.0, pH 8.0) as demonstrated in Table 1. (Y) was selected as a dependent variable to represent total polyphenol content (TPC).

Table 1. Actual and coded levels of the extraction variables selected for Box-Behnken Design optimization

Independent variables	Codes	Variable levels			Dependent variables	Goal
		-1	0	1		
Temperature	X_1	40°C	55°C	70°C	Y: Total polyphenol content (mg GAE/g dw)	Maximize
Time	X_2	60 mins	90 mins	120 mins		
pH	X_3	6.0	7.0	8.0		

Determination of total polyphenol content

The total polyphenol content was determined using the FC method and gallic acid as a standard in different concentrations ranging from 15.625 - 125 µg/mL in a 96-well plate. Briefly, a 30 µL diluted sample was added to 62.5 µL FC reagent 10% (w/v), incubated for 5 minutes in dark before subsequently adding 60 µL sodium carbonate (Na_2CO_3) solution 7.5%. The absorbance of the mixture was measured at 765 nm using a UV spectrophotometer after incubation for 45 minutes at room temperature [22]. Blank contained all reagents except for the sample extract. The concentrations of polyphenol in the extract were determined by gallic acid standard calibration (y = 0,0087x + 0,0346, R^2 = 0,9995). The total polyphenol content was expressed as milligram gallic acid equivalent (mg GAE/g dw) and was calculated according to the equation [22]:

(1) TPC (mg/g) =

$$\frac{Concentration\ of\ polyphenol\ in\ the\ extract\ \left(\frac{mg}{\mu L}\right)\ x\ Volume\ (mL)}{sample\ dry\ weight\ (g)}$$

Ferric reducing antioxidant power (FRAP) assay

FRAP activity of *Perilla* extract was evaluated using the Oyaizu method that referred to the standard calibration curve of gallic acid (y = 0,0002x + 0,089, R^2 = 0,9879) [22]. Gallic acid solutions of 62.5, 125, 250, 500, and 1000 µg/mL were prepared as standard solutions and the results were expressed as mg gallic acid equivalent (GAE)/g dw. Twenty µL of standards/ samples were added to eppendorfs containing 62.5 µL phosphate buffer saline (PBS), 62.5 µL $[K_3Fe(CN)_6]$ 1% solution, then mixed well and allowed to react for 20 minutes at 50°C without light. Then adding 62.5 µL TCA (10% w/v) and 3 µL $FeCl_3$ (0.1% w/v) [6], before the absorbance was measured at 700 nm thanks to Prussian blue formation (ferric ferrocyanide) through the reaction between ferric ion and dilute potassium ferrocyanide solution [23].

Genomic DNA preparation

Escherichia coli genomic DNA was extracted by adopting a procedure described by Ghatak et al. with some modifications [24]. Firstly, the stock was centrifuged at 8000 rpm, 25°C for 10 minutes to obtain the pellet, then repeated centrifugation twice with distilled water. The pellet subsequently was resuspended in a 500 µL mixture of solution I (50 mM glucose, 10 mM EDTA, and 25 mM Tris-HCl, pH 8.0) and lysozyme (ratio Lysozyme: Solution I: 1: 1000). After 30-minute incubation at 37°C, solution II containing 1% SDS and 20 mM NaOH was added to the mixture, followed by a further 30-minute incubation at 55°C. Three hundred µL CH_3COONa 3M was introduced and then centrifuged at 8000 rpm for 15 minutes to precipitate protein and obtain DNA supernatant only. Prior to overnight chilling in a 4°C refrigerator, a volume that was twice as large as the volume of absolute EtOH's supernatant was added. Finally, the mixture was centrifuged at 8000 rpm for 30 minutes to obtain pellets containing *E. coli* DNA. DNA with TE buffer was stored at -20°C for further tests.

DNA protection assay

E. coli genomic DNA was used to perform inhibition potential against DNA damage of *P. frutescens* extracts. Thirty µL of DNA, different

pISSN 1859-1388
eISSN 2615-9678

plant extractions, and 30% H_2O_2 were prepared corresponding to Table 2 and Table 5 [25]. For experiment A, all substances were inserted into the eppendorfs simultaneously. For experiment B, the mixture of genomic DNA and plant extract was added first for 30-minute incubation at room temperature before introducing H_2O_2. A tube containing DNA and H_2O_2 without extracts was prepared as a negative control and positive control was a tube with only DNA. All were exposed to UV light at a wavelength of 365 nm for 2 hours. After irradiation exposure, the presence of DNA was confirmed by agarose gel electrophoresis (0.7%) and dsDNA concentration was measured at 260 nm wavelength using Thermo Scientific™ NanoDrop™ One Microvolume UV-Vis Spectrophotometers.

Table 2. The amount of DNA, H_2O_2, plant extracts (µL) and time of incubation (min) for each sample in DNA protection assay. N (Negative): DNA + H_2O_2; P (Positive): untreated DNA; A4, A10, A11: DNA + Extract No. 4, 10, 11, respectively + H_2O_2 without incubation; B4, B10, B11: DNA + Extract No. 4, 10, 11, respectively (30 minutes) + H_2O_2

	Control		A			B		
	N	**P**	**No.4**	**No.11**	**No.10**	**No.4**	**No.11**	**No.10**
DNA (µL)	10	10	10	10	10	10	10	10
Extract (µL)	0	0	10	10	10	10	10	10
Incubation time (min)	0 min	0 min	0 min	0 min	0 min	30 min	30 min	30 min
H_2O_2 (µL)	10	0	10	10	10	10	10	10
TEB (µL)	10	0	0	0	0	0	0	0

Statistical data analysis

All experiments were performed in triplicate (n=3) and their means ± SD (standard deviation) were used for data analysis carried out by Box-Behnken Design in Design Expert software (version 12.0.3.0) and Microsoft excel. Analysis of variance (ANOVA) was used to analyze the results statistically.

3 Results

Optimization of total polyphenol content (TPC) BBD/RSM responses to TPC extraction

Table 3 illustrated the BBD matrix that each row presented for each run of the experiment including coded and corresponding actual values of three independent variables, total polyphenol contents (TPC), and ferric reducing antioxidant power (FRAP). The BBD generated 15 experiment runs in total consisting of 3 central points. Total polyphenol yields varied from 6.056 ± 0.08 to 9.630 ± 0.127 (mg GAE/g dw).

Table 3. Box–Behnken design and experimental data of total polyphenol and antioxidant activity based on FRAP from *P. frutescens*

No.	X_1	X_2	X_3	Temp t (°C)	Time (min)	pH	TPC (mg GAE/g) ± SD	FRAP (mg GAE/g) ± SD
1	0	1	1	55	120	8	7.201 ± 0.081	3.21 ± 0.019
2	0	1	-1	55	120	6	7.497 ± 0.033	4.05 ± 0.015
3	0	-1	1	55	60	8	6.180 ± 0.088	3.90 ± 0.017
4	**0**	**-1**	**-1**	**55**	**60**	**6**	**7.443 ± 0.056**	**4.68 ± 0.025**
5	1	0	1	70	90	8	8.943 ± 0.012	2.64 ± 0.034
6	-1	0	1	40	90	8	6.056 ± 0.080	2.85 ± 0.019
7	1	0	-1	70	90	6	8.032 ± 0.037	3.15 ± 0.032
8	-1	0	-1	40	90	6	6.291 ± 0.085	3.45 ± 0.025
9	1	1	0	70	120	7	9.625 ± 0.073	3.21 ± 0.030
10	-1	1	0	40	120	7	7.109 ± 0.016	3.36 ± 0.028
11	**1**	**-1**	**0**	**70**	**60**	**7**	**9.630 ± 0.127**	**3.69 ± 0.021**
12	-1	-1	0	40	60	7	6.642 ± 0.031	3.96 ± 0.037
13	0	0	0	55	90	7	6.234 ± 0.075	3.36 ± 0.011
14	0	0	0	55	90	7	6.697 ± 0.038	3.30 ± 0.018
15	0	0	0	55	90	7	6.775 ± 0.14	3.45 ± 0.083

As shown in Table 3, the average TPC of all extracts recorded was 7.399 mg GAE/g dw. The maximum yield of phenolic content was at 9.630 ± 0.127 mg GAE/g dw which was extracted under conditions 70°C, 60 min, and pH 7.0. The sample

extracted at 40°C, 90 min, and pH 8.0 was the lowest at 6.056 ± 0.080 mg GAE/g dw.

As mentioned previously, Table 4 displayed a high R^2 value (0.9535), a small difference between R^2 and adjusted R^2 (close to 1), a non-significant lack of fit (p>0.05), and a significant p-value (0.0077) for predictive models. Thus, the model was well-fitted with experimental data and can be reliable to predict the values of total polyphenols. RSM analysis generated a regression equation to represent the correlation between extraction yields and extraction factors:

(2) **TPC** = 22.69257 – 0.498015 × Temp – 0.179078 × Time + 0.972667 × pH – 0.000262 × Temp × Time + 0.0191 × Temp × pH + 0.008083 × Time × pH + 0.004294 × $Temp^2$ + 0.000796 × $Time^2$ – 0.204333 × pH^2

Following the regression equation (2) built by the optimization tool in the Design-Expert, the optimal point for maximum predicted phenolic content was found to be 9.807 mg GAE/g dw extracting at 70°C, 120 min, and pH 8.0 and it can be observed in Figure 2.

Influence of extraction factors on TPC and fitting of prediction models

Table 4 described the BBD matrix in which each experiment run was represented in a row along with its results (total phenolic content, Y). For each response, F-value, P-value, and coefficient regression were calculated. Analysis of variance (ANOVA) was used to determine the model's significance. For each model term (linear, interactive, and quadratic terms), a larger F-value and a smaller P-value indicated a greater influence on the corresponding response variables. The importance of each coefficient was evaluated using the P-values, which also showed the strength of each parameter's interaction with the responses.

Table 4. Analysis of Variance (ANOVA) for Quadratic model of Total polyphenol content (TPC) and Ferric reducing antioxidant power (FRAP) activity and Coefficients

Source	TPC			FRAP		
	Coefficient estimate	P-value	F-value	Coefficient estimate	P-value	F-value
Adjusted R^2		0.8698			0.9599	
R^2		0.9535			0.9857	
Model		0.0077 < 0.05	11.39		0.0004 < 0.05	38.19
X_1	1.27	0.0004 < 0.05	68.47	-0.1163	0.0235 < 0.05	10.37
X_2	0.1925	0.2641	1.58	-0.3000	0.0004 < 0.05	69.06
X_3	-0.1100	0.5045	0.5165	-0.3412	0.0002 < 0.05	89.36
X_1X_2	-0.1180	0.6091	0.2972	0.03	0.5823	0.3453
X_1X_3	0.2865	0.2429	1.75	0.0225	0.6778	0.1942
X_2X_3	0.2425	0.3135	1.26	-0.0150	0.7807	0.0863
X_1^2	0.9662	0.0078 < 0.05	18.39	-0.3762	0.0009 < 0.05	50.14
X_2^2	0.7167	0.0245 < 0.05	10.12	0.5613	0.0001 < 0.05	111.57
X_3^2	-0.2043	0.4060	0.8225	0.0287	0.6117	0.2928
Lack of fit		0.2609	2.99		0.3094	2.38

pISSN 1859-1388
eISSN 2615-9678

According to Table 4, the prediction model based on the output of the design was significant and suitable for use in this experiment due to a P-value result of 0.0077 (<0.05) and an F-value of 11.39. These values also illustrated that there was only a 0.77% chance the difference caused by noise (this large F-value) could occur which can negatively affect the prediction values. In addition, the "Lack of fit " in statistics showed how large the variation of the design points about their predicted values was when compared to the variation of the experimental replicates about their mean values. The P-value of "lack of fit" was insignificant (p = 0.2609 >0.01); thereby, the non-significant lack of fit indicated that the design model was sufficient for predicting the responses. Therefore, it could be concluded that the prediction model was reliable enough to navigate the experiment and evaluate the factors that affect the dependent responses.

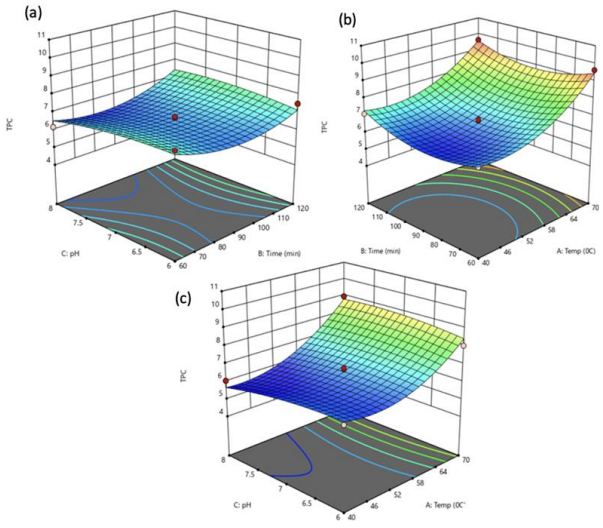

Fig. 1. Response surface model 3D plots showing the effects of Temperature (°C), Time (min), and pH on TPC. (a) interaction between Time and pH, (b) interaction between Time and Temperature, (c) interaction between Temperature and pH

The linear parameters – Temperature (X_1) had a significant impact on the response as its P-value was < 0.05 and F-value was 68.47. Besides, with a regression coefficient = 1.27, the temperature was considered as the greatest magnitude that affected the TPC yield (Y). The positive sign of the regression coefficient indicated that the polyphenol yield increased with the increasing temperature. In addition, there were directly proportional effects on the extraction process of the quadratic terms of temperature and extraction time (X_1^2 and X_2^2) with p<0.05 and regression coefficients = 0.9662 and 0.7167, respectively. All other factors having p>0.05 including pH, interactive terms (X_1X_2, X_1X_3, X_2X_3), and a quadratic term of pH (X_3^2) all had no significant effect.

Figure 2 was a three-dimensional (3D) response surface plot exhibiting the important collaborative effects caused by each pair of factors. Each panel displayed the effect of two factors on the extraction yields, while the third component was kept constant at 55°C for (a), pH 7.0 for (b), and 90 min for (c). As illustrated in Figure 2, pH had no significant influence on the extraction process of polyphenols from *P. frutescens.* In contrast, temperature extraction showed the most remarkable influence on TPC obtained which was directly proportional to the rise in temperature. Although the polyphenolics relatively reached the highest number at the points of 60 min and 120 min, it gradually decreased over a period of time from 70 min to 100 min.

Optimization of Ferric reducing antioxidant power (FRAP) activity BBD/RSM response of FRAP assay

According to Rahman, the higher absorbance of the reaction mixture (the higher concentration of FRAP), the higher the reducing capacity [12]. Table 3 showed the reducing power activity of *P. frutescens* within a range from 2.64 ± 0.34 to 4.68 ± 0.25 mg GAE/g dw. The mean recorded value was 3.484 mg GAE/g dw. The highest value documented (4.68 ± 0.25 mg GAE/g dw) was from

extract having 7.443 mg GAE/g dw which was extracted under conditions of 55°C, 60 min, and pH 6.0. In contrast, the extract containing TPC equal to 8.943 mg GAE/g dw had the lowest metal-reducing power (2.64 ± 0.034 mg GAE/g dw) that was extracted at 70°C, 90 min, and pH 8.0.

The regression equation in terms of actual parameters can be used to make predictions about the response of FRAP activity for given levels of each factor:

(3) **FRAP** = 9.07903 + 0.159694 × Temp – 0.122417 × Time – 0.78125 × pH + 0.000067 × Temp × Time + 0.0015 × Temp × pH – 0.0005 × Time × pH – 0.001672 × $Temp^2$ + 0.000624 × $Time^2$ + 0.02875 × pH^2

BBD - RSM analysis on the Design-Expert software was used to predict the most optimal parameter values for maximizing desired antioxidant capacity. Based on the prediction of the models (regression equation (3)), the optimized conditions calculated with the highest capacity in ferric reducing power were a temperature of 51.65°C, a time of 60 min, and pH 6.0 to obtain 4.605 mg GAE/g dw. Table 4 displayed the approximation between experimental R^2 of 0.9867 and adjusted R^2 of 0.9599. That also indicated the reliability of the predicted model and the model can be used to navigate the experiments.

Influence of extraction factors on FRAP and fitting of prediction models

As aforementioned, the smaller the P-values and the bigger the F-values are, the more significant the corresponding coefficient. Following Table 4, the prediction model based on the experiment response (FRAP) was significant and appropriate for use in this experiment, with a small P-value of 0.0004 (<0.05) and a large F-value of 38.19. A non-significant lack of fit (p>0.05) indicated that the predicted model matched the experimental data well since the variation of the replicates to their experimental mean values was not significantly different from the variance of the design points about their predicted values. From this result, it was possible to conclude that the prediction model was appropriate for predicting and assessing the factors influencing the dependent responses FRAP.

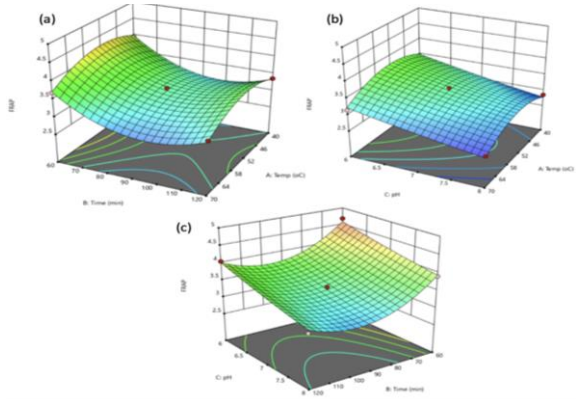

Fig. 2. Response surface model 3D plots showing the effects of Temperature (°°C), Time (min), and pH on FRAP activity. (a) interaction between Time and Temperature, (b) interaction between pH and Temperature, (c) interaction between Time and pH

The effects of three factors and their interaction on the FRAP value of phenolic extract can be seen in Table 4. The ANOVA on regression coefficient revealed the parameters had significant effects (p < 0.0001) on the result of FRAP including all three linear temperature (X_1), time (X_2), pH (X_3), and quadratic effects of temperature (X_1^2), and time (X_2^2). Meanwhile, interaction parameters of X_1X_2, X_1X_3, and X_2X_3 and a quadratic term of pH (X_3^2) did not show an insignificant influence. The negative sign of linear parameters (temperature (X_1), time (X_2), pH (X_3), and quadratic parameter (X_1^2) implied that the ferric-reducing ability had inversely proportional to these parameters. In other words, the increase in those factors led to a decline in FRAP activity. The regression coefficient (0.0246) of the quadratic time

pISSN 1859-1388
eISSN 2615-9678

parameter showed the most significant effects due to its highest value.

As shown in Figure 3, when the temperature was at 40°C and 70°C, the FRAP activity of polyphenols decreased to the lowest numbers. While the extraction temperature was heated up to the range of 51°C - 55°C, the FRAP values reached the highest (Figures 3(a) and 3(b)). The result of this study also showed that the reducing power of polyphenols was recorded to gradually increase when extraction pH was reduced from 8.0 to 6.0 (inverse proportion). Furthermore, the enhancement of FRAP activity was significantly influenced by the time of the process in which reducing power reached maximum ability at the first 60 min, then gradually dropped within the period of 70 min to 105 min. At 120 min, the figure showed a slight growth of FRAP activity; however, it was lesser than the 60-min one (illustrated in figures 3(a) and 3(b)).

DNA protection activity of different extracts of *P. frutescens*

Once performed with *E. coli* genomic DNA, the potential in DNA protection of TPC from *P. frutesccens* against free-radical damage was confirmed (Figure 3). The presence of *E. coli* DNA band intensity in all lanes indicated that there was a protective effect of TPC, except for the negative (Lane N). The concentration of DNA stock was equal to the concentration of untreated *E. coli* DNA (393.5 ± 3.30 ng/µL) which was a positive control (Lane P). In the negative control sample without extract, no DNA band appeared on the agarose gel, and the concentration of remaining DNA after the damage process was only 130.4 ± 2.34 ng/µL in comparison to the positive one (393.5 ± 3.30) (Table 6).

Table 5. The selected conditions to screen for DNA protection ability

No.	Tempt (°C)	Time (min)	pH	Experiment TPC (mg GAE/g dw) ± SD	FRAP (mg GAE/g dw) ± SD
4 (highest FRAP)	55	60	6	7.443 ± 0.056	4.68 ± 0.025
11 (highest TPC)	70	60	7	9.630 ± 0.127	3.69 ± 0.021
10	40	120	7	7.109 ± 0.016	3.36 ± 0.028

Table 6. dsDNA concentration after the process of H_2O_2 and UV light damage (ng/µL)

	Extract No.4	Extract No.11	Extract No.10	Negative control	Positive control
A (0 min)	206.7 ± 2.33	180.2 ± 1.98	192.5 ± 3.87	130.4 ± 2.34	393.5 ± 3.30
B (30 min)	215.3 ± 3.49	178.8 ± 4.43	187.6 ± 3.74		

Different levels of protection from OH radical damage were offered by different *P. frutescens* extracts. Through ANOVA, a p-value of incubation time was bigger than 0.05 which indicated the insignificant effect of this factor on the activity of DNA protection. In other words, there were no significant differences between sample A (simultaneously mixed) and sample B (mixed DNA and extract for 30 minutes before introducing H_2O_2).

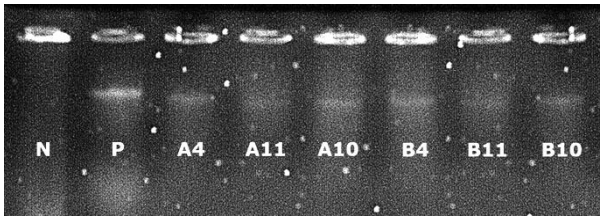

Fig. 3. Agarose gel electrophoresis of *E. coli* DNA protected by *P. frutescens* extracts against H_2O_2 and UV light damage. (N): DNA + H_2O_2; (P): untreated DNA; A4, A11, A10: DNA + extract + H_2O_2 simultaneously, B4, B11, B10: DNA + extract (30 minutes) + H_2O_2

According to Figure 3, the DNA ran from the wells (negative electrode) towards the positive electrode (from the top to the bottom). The more intense staining of that band, the more double-stranded DNA in a band, and also the closer the DNA bands were to the wells, the larger the remaining DNA size [27]. This result displayed that extract No.4, with the strongest antioxidant ability by reducing free radicals, had the greatest potential to protect DNA from H_2O_2 and UV light damage (residual DNA concentrations after assay were equal to 206.7 ± 2.33 and 215.3 ± 3.49 ng/µL). Moreover, sample No.11 with the highest TPC result (9.630 mg GAE/g dw) showed the lowest value of DNA concentration. It was lower than sample No.10 with the lowest experimental TPC and FRAP results among the 3 selected samples.

4 Discussion

Optimization of TPC

Box–Behnken design (BBD) of Response Surface Methodology was used to analyze and optimize the influence of 3 extraction factors (temperature, time, and pH) on the total polyphenol contents (TPC). As temperature affected the extraction potency and the quality of the components in the extract, the temperature was selected in the optimization study. Moreover, the extraction process at a suitable temperature is not enough to ensure the yield and the component quality, the extraction process required time optimization for extraction. Therefore, the study focused on temperature and time factors in the optimization of extraction. Although there are many various factors influencing extraction potency, pH was selected in the study. Certainly, some components in the extract changed their activities under alkali, neutral or acidic conditions. It might be the reasons that three factors were used in the study. Using RSM, the study will detect the multi-factors influencing the extraction potency conveniently when the experimental matrix was established for the study. Moreover, the interaction of three factors was studied whether they affect the extraction, which was better than one-factor optimization. Additionally, the obtained results were analyzed when using RSM and Box-Behnken design. Because the number of experimental runs for this design was significantly smaller than other factorial techniques, this is a cost and time-saving method for optimization. Furthermore, Box-Behnken predicts more precision in the center of the factor space for optimization [15]. Only temperature (X_1) and quadratic terms of temperature and extraction time (X_1^2 and X_2^2) had a directly proportional effect on the extraction process. This meant that the increases in TPC followed the increases in these variables and temperature gave the most significant effect. The optimum conditions generated by Design Experts were an extraction temperature of 70°C, an extraction time of 120 min, and a pH of 8.0 to obtain 9.807 mg GAE/g dw. As a result, pH had no significant effect on the yields of TPC in the extract. The sample with conditions 70°C and 120 min, pH 7.0 obtained 9.625 mg GAE/g dw TPC, which was slightly lower than the experimental sample extracted at 70°C, 60 min, and pH 7.0 (9.63 mg GAE/g dw). Therefore, a solution suggested to validate the predicted optimal point is to perform the Folin-Ciocalteu method for the extract following predicted optimal conditions. The

pISSN 1859-1388
eISSN 2615-9678

prediction and experimental data are going to compare using a statistical analysis paired comparison T-test. In addition, the experimental TPC was shown that samples extracted at 60 min and 120 min had high concentrations of TPC, and yet a decline at 70 min and 100 min. According to a study, the probability of oxidation, epimerization, and degradation of bioactive chemicals increases with extraction duration [25]. To explain the decrease in TPC yields from 70 min to 100 min, potentially, each type of polyphenolic chemical contained in *P. frutescens* extract was formed and broken down at different time intervals. In other words, *Perilla* extract may contain polyphenol compounds that were only extracted when times were at 60 min and 120 min. Some compounds were degraded during a period from 70 min to 100 min when continuously exposed to the temperature. This is because polyphenols consist of various chemical components, such as catechins, proanthocyanidins, anthocyanins, gallotannins, ellagitannins, flavonol glycosides, hydroxycinnamoyl esters, lignoids, and stilbenoids. These polyphenol subclasses have distinct chemical reactivities. To verify this hypothesis, additional tests need to be conducted under the optimal conditions identified (60 min and 120 min).

Optimization of FRAP activity

In evaluating the antioxidant activity of plant polyphenols, reducing power is also widely used. Polyphenols are one of the most important plant phytochemicals with antioxidant activity due to their redox capabilities, such as scavenging and neutralizing free radicals, quenching singlet and triplet oxygen, and dissolving peroxides [26]. As shown in Table 4, all linear independent parameters had a significant impact on FRAP, and all extracts in this study possessed good reducing properties, indicating antioxidant activity as well. Thanks to Design Expert software, the optimized condition (4.605 mg GAE/g dw) was calculated, comprising a temperature of 51.65°C, a time of 60 min, and a pH of 6.0. In comparison to the result of LI et al., the FRAP of *Perilla* leaves extracted with ethanol using ultrasound-assisted extraction was highest at conditions of 54°C and 52 min [37]. There are papers that reported the antioxidant capacity of extracts increases along with the increase in the TPC [12]. However, sample 11 with the highest TPC (9.630 mg GAE/g dw in TPC) in this project had a lower iron-reducing ability than sample 4 (7.443 mg GAE/g dw in TPC) with the highest FRAP. This was because the levels of antioxidant activity of polyphenols depend on many other variables. According to Bayliak MM , TPC may act as an effective antioxidant at acidic pH rather than an alkaline one [28] and acidic pH also contribute to the preservation of polyphenol compounds and antioxidant properties during the storage process [34]. For example, in acidic solutions, anthocyanins are more chemically stable [36]. Besides, the efficiency of polyphenols in antioxidants also relies on types of polyphenols in extracts and their chemical structures. In other words, phytochemical constituents (apigenin, catechin, and malonylshisonin), the number of hydroxyl groups and structure in their molecules also affect the efficiency of polyphenols in antioxidant and iron-reducing ability [29]. Furthermore, different *Perilla* cultivars contain different types of phenolic compounds in various amounts that confer different levels of bioactivities. Therefore, the correlation between the total polyphenol content of *P. frutescens* and ferric-reducing antioxidant power is more complicated and requires further investigation for deeper understanding.

DNA protection activity against free radical damage

The production of OH radicals through UV-photolysis and H_2O_2 is a major cause of most protein and DNA oxidative damage. In this study, the DNA protection assay was used as a rapid and reliable method that offered a straightforward method to demonstrate the protective effect in vitro [30].

The experiment performed with *E. coli* indicated the differences between extracts and incubation times. In particular, all 3 extracts used to screen for DNA protection displayed considerable protective activity against free radical DNA damage as well as antioxidant capacity (Figure 3). The non-significant difference between A and B (p>0.05) implied that longer exposure to extracts containing polyphenols did not effectively enhance DNA protection activity. The residual DNA after damage with H_2O_2 and UV light of A4 and B4 samples was 206.7 and 215.3 ng/µL, respectively. This result noted that the reducing power was proportional to the protective DNA activity. Two samples 10 and 11 showed relatively close amounts in the number of remaining DNA concentrations even though containing higher and lower values in polyphenolic content. Therefore, different conditions of extraction would offer different quantities of TPC and polyphenolic components that gave different levels of protection against DNA oxidative damage. The study used *E. coli* to study because there was the previous study on DNA damage prevention due to Dbp (DNA binding protein) action [31]. This protein helped *E. coli* avoid near ultraviolet rays. However, our study damaged DNA under ultraviolet rays (365 nm) (Figure 4). To confirm DNA damage in *E. coli*, we cultured bacterium on the medium to check the growth. There was no colony recovered on the medium. DNA of *E. coli* could not be prevented by Dbp, but it might be prevented by other mechanisms. The study did not test on human DNA because human DNA is easy to damage under ultraviolet rays, however, DNA of *E. coli* is more stable [30]. Therefore, the study used DNA of *E. coli* as a model for study. When DNA of *E. coli* is degraded under the effects of ultraviolet rays and the extract can protect the DNA of *E. coli*, it can support the beneficial effects in human DNA. Moreover, in case we tried to work with human products, the ethical policy is required. Antioxidant activity of the plant extract could play a role in DNA damage protection. The study suggested the importance of *P. frutescens* (L.) Britt in human care should be more exploited.

5 Conclusion

The study found that the temperature of 70°C is the optimal conditions to obtain the highest polyphenol content in the extraction of *P. frutescens* (L.) Britt and it also had the most significant effect on the results. Furthermore, this research proposed that *P. frutescens* (L.) Britt is a good source showing DNA protection and antioxidant activity at certain concentrations. The experiment was conducted on bacterial genomes, not on human genomes. If possible, the experiment on DNA protection of TPC should be validated on the human genome to increase its application in pharmaceutical and related fields. However, the study supplied the way to get a high yield of polyphenol with antioxidant and DNA protection activities. This study is still being continued for future application.

Competing interests

The authors declare that there was no conflict of interest regarding the publication of this article.

pISSN 1859-1388
eISSN 2615-9678

References

1. Sies H. Strategies of antioxidant defense. European Journal of Biochemistry. 1993;215(2):213-219.
2. Buechter DD. Free radicals and oxygen toxicity. Pharmaceutical Research. 1988;5(5):253-260.
3. Young IWJ. Antioxidants in health and disease. Journal of Clinical Pathology. 2001;54:176-186.
4. Valko MRC. Free radicals, metals, and antioxidants in oxidative stress-induced cancer. Chemico-Biological Interactions. 2006;160:1-40.
5. Wulf D. Free radicals in the physiological control of cell function. Physiological Reviews. 2002;82:47-95.
6. Willcox JKAS. Antioxidants and prevention of chronic disease. Critical Reviews in Food Science and Nutrition. 2004;44:275-295.
7. Koçyiğit A. Eksojen Antioksidanlar İki Yönü Keskin Kılıçlardır. Bezmialem Science. 2016;4(2):70-75.
8. Ito NHM. Studies on antioxidants; their carcinogenic and modifying effects on chemical carcinogenesis. Food and Chemical Toxicology. 1986;24:1071-1081.
9. Sasaki YFKS. The comet assay with 8 mouse organs: results with 39 currently used food additives. Mutation Research-Genetic Toxicology and Environmental Mutagenesis. 2002;519:103-119.
10. Rajkumar VGG. Evaluation of antioxidant activities of *Bergenia ciliata* rhizome. Records of Natural Products. 2010;4(1):38-48.
11. Pizzino GI. Oxidative stress: Harms and benefits for human health. Oxidative Medicine and Cellular Longevity. 2017; 1-13.
12. Rahman MM. Effect of freeze drying on antioxidant activity and phenolic contents of Mango (*Mangifera indica*). International Food Research Journal. 2015;22(2):613.
13. Heci Y. Valuable ingredients from herb perilla: A mini-review. Innovations in Food Technology. 2001;29:32-33.
14. Meng L, Lozano Y, Bombarda I, Gaydou E, Li B. Polyphenol extraction from eight *Perilla frutescens* cultivars. Comptes Rendus Chimie. 2009;12:602-611.
15. Rajasimman ND. Optimization and kinetics studies on biodegradation of atrazine using mixed microorganisms. Alexandria Engineering Journal. 2013;52(3):499-505.
16. EA D. Phenolics: Prooxidants or antioxidants?. Nutrition Reviews. 1997;55(11):396-398.
17. Rajbhar KA. Polyphenols: Methods of extraction. Scientific Reviews and Chemical Communications. 2015;5(1):1-6.
18. Maisuthisakul P, Pongsawatmanit R, Gordon MH. Characterization of the phytochemicals and antioxidant properties of extracts from Teaw (*Cratoxylum formosum* Dyer). Food Chemistry. 2007;100(4):1620-1629.
19. Zimeri J, Tong CH. Degradation Kinetics of (−)-Epigallocatechin Gallate as a Function of pH and Dissolved Oxygen in a Liquid Model System. Journal of Food Science. 1999;64(5):753-758.
20. Zeng L, Mẹngun M, Chen L, Liyong L. Stability of tea polyphenols solution with different pH at different temperatures. International Journal of Food Properties. 2017;20(1):1-18.
21. He B, Zhang LL, Yue XY, Liang J, Jiang J, Gao XL, et al. Optimization of ultrasound-assisted extraction of phenolic compounds and anthocyanins from blueberry (*Vaccinium ashei*) wine pomace. Food chemistry. 2016;204:70-76.
22. Shao PH. Analysis of conditions for microwave-assisted extraction of total water-soluble flavonoids from *Perilla frutescens* leaves. Journal of food science and technology. 2012; 49(1):66-73.
23. Saifullah MM. Optimization of microwave-assisted extraction of polyphenols from Lemon Myrtle: Comparison of modern and conventional extraction techniques based on bioactivity and total polyphenols in dry extracts. Processes. 2021;9(12):2212.
24. Siddiqui NR. Spectrophotometric determination of the total phenolic content, spectral and fluorescence study of the herbal Unani drug Gul-e-Zoofa (*Nepeta bracteata* Benth). Journal of Taibah University Medical Sciences. 2017;12(4):360-363.
25. Oyaizu M. Antioxidative activities of browning reaction prepared from glucosamine. The Japanese Journal of Nutrition and Dietetics. 1986;44:307-315.
26. John DB. Theory and Practice of Histological Techniques (Sixth Edition). Elsevier Health Sciences; 2008.
27. Bayliak MM. Effects of pH on antioxidant and prooxidant properties of common medicinal herbs. Open Life Sciences. 2016;11(1):298-307.

28. Ghatak SM. A simple method of genomic DNA extraction from human samples for PCR-RFLP analysis. Journal of Biomolecular Techniques. 2013;24(4):224-231.

29. Adinortey MBAC. DNA Damage Protecting Activity and Antioxidant Potential of *Launaea taraxacifolia* Leaves Extract. Journal of Natural Science, Biology and Medicine. 2018;9(1):6-13.

30. Vuong QV, Golding JB, Stathopoulos CE, Nguyen MH, Roach PD. Optimizing Conditions for the Extraction of Catechins from Green Tea Using Hot Water. Journal of Separation Science. 2011;34:3099-3106.

31. Calderon-Montano M, Burgos-Moron E, Perez-Guerrero C, Lopez-Lazaro M. A Review on the Dietary Flavonoid Kaempferol. Journal of Medicinal Chemistry. 2011;11:298-344.