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Article

Optimization of Ultrasound-Assisted Extraction on Antioxidative Activity of *Malus toringoides* Using Response Surface Methodology

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Abstract: Ultrasonic-assisted extraction (UAE) was optimized using response surface methodology (RSM) to maintain the cyto-protective activity of *M.toringoides* against oxidative stress. The optimal conditions for UAE were a 58 mL/g liquid-solid ratio, a 38 °C extraction temperature, an 85% solvent concentration, and a 19-min extraction time, which resulted in a protection rate of 54.57% against hydrogen peroxide-induced oxidative stress in human umbilical vein endothelial cells (HUVECs). These results were comparable to the predicted value of 53.75%. The extracts showed excellent antioxidant activity, and phlorizin was detected in the dried leaves of *Malus.toringoides*. The highest yield of phlorizin (101.239 mg/g) was also obtained using these conditions. Taken together, these results showed that the method successfully integrated RSM and partial least squares regression methods to optimize *M.toringoides* extraction to yield the highest cyto-protective activity and effectively increase the yield of phlorizin from *M.toringoides*.

Keywords: *M.toringoides*; human umbilical vein endothelial cells; ultrasonic-assisted extraction; protection rate; response surface methodology

1. Introduction

The leaves of *M.toringoides* are a source of traditional folk medicine in Tibet, China. The traditional medicine is obtained from the leaves of *M.toringoides* (Rehd.) Hughes or *Malus tiansitoria* (Batal.) Schneid, which grow on snowy mountains at an attitude of 3000 to 3700 m and has been used as a food source. The active compounds include phlorizin, phloretin, quercetin, isoquercitrin, rutin, and hyperin as well as others. It has been used in the treatment of hypertension, indigestion, liver injury, hyperlipidaemia, and hyperglycemia [1]. Various substances isolated from *M.toringoides* leaves have strong antioxidant activities, but the antioxidant activity of *M.toringoides* has not been formally researched [2]. Processing of *M.toringoides* is currently dependent upon experience, which results in a low efficiency and unstable quality of *M.toringoides* extractions.

Ultrasound assisted extraction (UAE) has been used for the extraction of valuable compounds from various natural sources [3–8]. UAE can enhance the extraction rate and reduce the processing

time [9–12]. When UAE was compared to conventional extraction methods, it was found to be a faster, more efficient, and solvent-saving technique [13–17]. Therefore, UAE is considered a convenient method for the extraction of bioactive compounds from food systems, and UAE methods can be optimized using mathematical models [18–20].

As a statistical experimental protocol, response surface methodology (RSM) is used to analyze empirical models that describe the effect of several independent variables on one or more dependent (response) variables, such as solvent concentration, extraction time, and their interactions on cyto-protective ability. To optimize the extraction by RSM that has been successfully employed, for example, polysaccharides and proteins from plants are optimized by RSM to determine extraction conditions that correlate with the highest biological activity [21,22]. However, few studies have been performed to optimize UAE to conserve cyto-protective effects via RSM.

Human umbilical vein endothelial cells (HUVECs) play an important role in humans, especially the regulation of cardiovascular health and the elasticity of vessels. Excessive accumulation of reactive oxygen species mainly induced oxidative stress, which is an important step in HUVEC injury [23,24]. Plants rich in antioxidants have attracted interest as strategies to counteract the injury caused by oxidative stress [25].

Therefore, the aim of this study was to optimize the conditions for rapid UAE of *M.toringoides* with high cyto-protective activity by using an RSM statistical approach and to compare the experimental and predicted protective effects. The extraction parameters (independent variables) included extraction time, solvent concentration, a solid-to-liquid ratio, and extraction temperature. Cell viability was assessed by measuring the cyto-protective effects on human endothelial cells against hydrogen peroxide (H₂O₂)-induced cell death using a cell viability assay. The cyto-protective assay was chosen to confirm the optimized extraction conditions. In addition, cyto-protective activity was correlated with antioxidant activity. To our knowledge, this is the first study showing optimization of the extraction processes of *M.toringoides* based on in vitro cyto-protective activity, as well as optimization of the active compounds in the extract.

2. Materials and Methods

2.1. Plant Material

Fresh *M.toringoides* was purchased from Ganzi Tibetan Autonomous Prefecture in October 2016 and identified by Professor Min Li, who is a pharmacognosist from the Department of Science of Identification of Chinese Materia Medica, School of Pharmacy, Chengdu University of Traditional Chinese Medicine (Chengdu, China). The plant was washed in distilled water and dried to remove the surface water. Then, the *M.toringoides* samples were dried to a constant weight.

2.2. Chemicals and Reagents

Several chemical reagents and solvents were used in this study. H₂O₂, ethanol, and MTT were purchased from Sigma Chemical Co. (St. Louis, MO, USA), PBS, DMEM media, and fetal bovine serum were purchased from HyClone Industries, USA. Trypsin was obtained from Biological Industries, Israel, and DMSO was obtained from MP Biomedicals.

2.3. High-Performance Liquid Chromatography (HPLC) Analysis of *M.toringoides*

Whole leaves from *M.toringoides* (0.5 g) were soaked in 40 times volume (*w/v*) of 70% ethanol for 20 min at 30 °C. The main components, which are phlorizin and phloretin, were quantified by LC-DAD analysis using an Agilent 1220 LC system. Chromatographic separation was performed using a Sciencelab Kromasil C18 column 250 × 4.6 mm² at 35 °C. A gradient elution was performed with solvent A (0.1% phosphate acid) and solvent B (acetonitrile), which varied the proportion of solvent B from 0 to 20% (0–30 min). The extract and the standard were dissolved in methanol and filtered through a 0.45-µm membrane (Millipore, Burlington, MA, USA) prior to injecting a volume of 20 µL.

2.4. Protection Rate of HUVECs

The HUVECs were a kind gift from Prof. HoSub Lee (WonKwang University, Iksan, Korea). The cells were cultured in DulbCo modified EAG medium (DMEM), containing 10% fetal bovine serum (Hyclone, Thermofisher, Beijing, China) and 1% penicillin/streptomycin, and cultured in a humidified environment of 5% carbon dioxide at 37 °C. Cell viability was determined by the colorimetric reagent 3-(4,5-dimethylthiazole-2-yl) -2,5-diphenyltetrazolium ammonium bromide (MTT). Human umbilical vein endothelial cells (HUVECs) were inoculated into 96-well plates at a density of 1.0×10^5 cells/mL to grow into fusion for 24 h. The cells were pre-treated with 100 µg/mL Begonia extract for 24 h, and then exposed to 0.2 mM of H₂O₂ for 4 h. As mentioned above, MTT was used to observe cell viability [26] after exposure to oxidative stress. MTT (5mg/mL) was added to each well and incubated at 37 °C for 4 h. After absorbing the medium, the purple precipitates formed in living cells were dissolved in dimethyl sulfoxide. The optical density of each well was measured with a microplate reader at 490 nm. The optical density of the control group was 100%.

$$\% \text{Protection rate} = (\text{OD}_{\text{positive control}} - \text{OD}_{\text{negative control}}) / (\text{OD}_{\text{control}} - \text{OD}_{\text{negative control}})$$

2.5. Single Factor Experiments and Response Surface Design

The major component of the *M.toringoides* extract was phlorizin, according to the HPLC results. Therefore, the phlorizin content and the cyto-protective activity are the important indexes to determine the quality of *M.toringoides* extracts. The extraction time, extraction temperature, solvent concentration, and solvent-solid ratio are key factors that affect the extract composition. Therefore, single factor experiments of these three influencing factors were carried out. The highest phlorizin contents in *M.toringoides* and cyto-protective activities in the four experiments were taken as the optimal central values. These values were subsequently used to study the best processing method for *M.toringoides* by RSM.

Whole leaves from *M.toringoides* (0.5 g) were soaked in 40 volume equivalents (mL/g) of 70% alcohol for 5 min, 10 min, 15 min, 20 min, and 25 min at 30 °C and filtered. Then, the cyto-protective activity of the extracts and the phlorizin contents were determined to obtain the best extraction time. Extraction temperature, solvent concentration, and the solvent-solid ratio were also obtained by this method.

2.6. Extraction Method

A total of 0.5 g of dried *M.toringoides* was weighed and transferred to a conical flask filled with ethanol. During ultrasound treatments, the desired temperature was kept constant throughout the trials. The experimental factors and their ranges, which were determined based on the preliminary single factor test runs, are given in Table 1.

Table 1. Experimental domain of the Box-Behnken design.

Independent Variable	Factor Levels		
	−1	0	1
X ₁ : Extraction time (min)	10	15	20
X ₂ : Extraction Temp (°C)	25	30	35
X ₃ : Solvent Concentration (100%)	70	80	90
X ₄ : Solvent-Solid Ratio (mL/g)	40	50	60

2.7. Experimental Design of Response Surface Methods

The single factor experiment was used to study the extraction process parameters in order to obtain the best central value of phloretin content. The optimum processing conditions, including phlorizin content and cell protection rate, were determined by BBD (design expert software, trial

version 8.0.5, Minneapolis Stat Ease Inc., Minneapolis, MN, USA). X1 (extraction time), X2 (extraction temperature), X3 (solvent concentration), and X4 (solvent-solid ratio) were selected as the main factors. The design consists of 29 experiments and 5 repetitive centers, which are used to estimate the sum of squares of pure errors. Phlorizin content and cell viability were Y1 and Y2, respectively.

3. Results and Discussion

3.1. The HPLC Analysis of *M.toringoides* Extracts

As illustrated in Figure 1, phlorizin was an abundant component of *M.toringoides* extracts. This compound is believed to be responsible for most of the pharmacological activities, including activities against hypertension, indigestion, liver injury, hyperlipidaemia, and hyperglycemia [27]. A previous study demonstrated the antioxidant effect of phlorizin [28]. Therefore, the phlorizin content in *M.toringoides* is an important index.

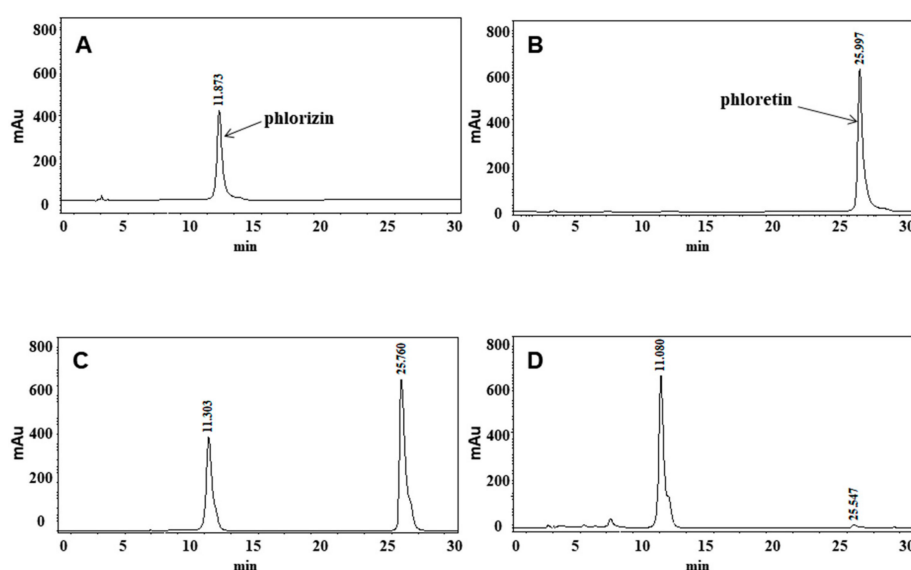


Figure 1. HPLC chromatogram of phlorizin (A). HPLC chromatogram of phloretin (B). HPLC chromatogram of phlorizin and phloretin (C). HPLC chromatogram of *M.toringoides* extract (D).

3.2. Analysis of Response Surface Model

The extraction time (10 and 20 min), extraction temperature (25 °C and 35 °C), solvent concentration (70% and 90%), and solvent-solid ratio (40 mL/mg and 60 mL/mg) were analyzed to determine the effect of the extraction parameters on the phlorizin content and cryo-protection of HUVECs against oxidative stress induced by hydrogen peroxide. The results are shown in Table 2.

Second order quadratic polynomial models were found to be adequate to describe the effect of independent factors on the protection rate, and the percent of phlorizin content was analyzed by the ANOVA method. The validation of the goodness of fit was determined by the lack of fit. The F-value was obtained from the lack of fit, and the p-value was obtained from the model. When the fitting of significant regression model ($p < 0.05$) and non-significant regression model ($p > 0.05$) is insufficient, the model agrees well with the experimental data. All the proposed mathematical models can be fully used to predict dependent variables as functions of independent variables. Extraction time (X_1), extraction temperature (X_2), solvent concentration (X_3), and the solvent-solid ratio (X_4) had significant linear effects on all reaction variables.

Table 2. BB design for actual factors and results for response surface methodology.

Run	X ₁ : Extraction Time (min)	X ₂ : Extraction Temp (°C)	X ₃ : Solvent Concentration	X ₄ : Solvent-Solid Ratio (mL/g)	Y ₁ : Phlorizin (mg/g)	Y ₂ : Protection Rate (%)
1	15	35	70	60	90.77	42.21
2	20	35	70	50	89.71	41.04
3	15	35	90	60	96.44	47.72
4	15	35	80	50	100.01	54.23
5	20	30	80	50	91.43	42.89
6	10	35	90	50	86.37	37.18
7	10	40	80	50	87.23	37.90
8	20	35	80	60	96.95	47.87
9	15	40	90	50	95.23	47.51
10	15	40	70	50	90.39	41.00
11	15	30	80	40	88.59	38.79
12	15	35	80	50	96.72	47.96
13	10	35	70	50	82.15	30.24
14	15	35	90	40	92.63	43.75
15	10	35	80	60	86.96	36.85
16	15	30	80	60	87.27	37.66
17	10	30	80	50	84.75	35.21
18	15	30	90	50	87.39	37.94
19	15	30	70	50	84.97	34.23
20	15	35	70	40	90.16	41.04
21	20	35	80	40	93.45	45.57
22	15	40	80	60	97.77	50.15
23	15	35	80	50	97.54	49.72
24	15	35	80	50	96.70	47.92
25	10	35	80	40	89.02	40.48
26	15	40	80	40	93.42	45.60
27	15	35	80	50	97.54	50.41
28	20	40	80	50	96.04	48.03
29	20	35	90	50	98.77	52.34

3.3. Effects of Extraction Conditions on Phlorizin Extracted from *M.toringoides*

The maximum phlorizin levels obtained from ultrasound assisted extraction (UAE) were 101.239 mg/g (18.82 min, 38.13 °C, 85.6%, and 59.43 mL/g). Based on ANOVA, the most dominant independent variable affecting the phlorizin content was the extraction time, and its F value was 98.3 ($p < 0.0001$). The effect of the three independent variables on the phlorizin content was expressed in quadratic terms (Table 3). The mathematical model can be expressed as follows.

$$Y_1 = 97.70 + 4.16X_1 + 2.97X_2 + 2.39X_3 + 0.74X_4 + 0.54X_1X_2 + 1.21X_1X_3 + 1.39X_1X_4 + 0.60X_2X_3 + 1.42X_2X_4 + 0.80X_3X_4 - 4.24X_1^2 - 4.03X_2^2 - 3.97X_3^2 - 1.66X_4^2 \quad (1)$$

As shown in Figure 2a–c, with the extension of extraction time, the yield percentage increased and reached a maximum at 15 min. Then the content slowly decreased. The positive linear effect of extraction temperature and its negative effect on the interaction with treatment time are shown in Figure 1a,d,e. The linear increase in phlorizin production may be due to higher system energy by higher temperature, which will enhance the release and solubility of the target compound [29]. Time/temperature interaction effect on the final result is clearly illustrated at the higher end of the temperature axis of the surface diagram, which indicates the adverse effects of long processing time (Figure 1a). In addition, as shown in Figure 2b,d,f, the relationship between higher yields and solvent concentration is due to an increased diffusion rate of solvents and solubility of phenazine. As shown in Figure 2c,e,f, the content of phlorizin is higher when the solvent-solid ratio increases from 40 mL/g to 50.00 mL/g. This increase in liquid-solid ratio leads to greater osmotic pressure and contact area,

which results in more solvents being pushed into plant substrates and facilitating the permeation of biologically active chemicals. The solvent enters through the cell wall. Empirical second-order polynomial model with experimental data applied to regression analysis.

Table 3. Analysis of variance for the fitted quadratic polynomial model for extraction of Phlorizin.

Source	Sum of Squares	df	Mean Square	F-Value	p-Value Prob > F	
Model	651.06	14	46.5	22.05	<0.0001	significant
X ₁ -Extraction time (min)	207.29	1	207.29	98.3	<0.0001	
X ₂ -Extraction temp (°C)	106.1	1	106.1	50.32	<0.0001	
X ₃ -solvent concentration (100%)	68.53	1	68.53	32.5	<0.0001	
X ₄ -Solvent-solid ratio (mL/g)	6.59	1	6.59	3.13	0.0988	
X ₁ X ₂	1.15	1	1.15	0.54	0.4732	
X ₁ X ₃	5.87	1	5.87	2.78	0.1176	
X ₁ X ₄	7.75	1	7.75	3.68	0.0758	
X ₂ X ₃	1.45	1	1.45	0.69	0.4209	
X ₂ X ₄	8.02	1	8.02	3.81	0.0714	
X ₃ X ₄	2.56	1	2.56	1.21	0.2893	
X ₁ ²	116.8	1	116.8	55.39	<0.0001	
X ₂ ²	105.59	1	105.59	50.07	<0.0001	
X ₃ ²	102.37	1	102.37	48.55	<0.0001	
X ₄ ²	17.97	1	17.97	8.52	0.0112	
Residual	29.52	14	2.11			
Lack of Fit	22.21	10	2.22	1.22	0.4603	not significant
Pure Error	7.31	4	1.83			
Cor Total	680.58	28				

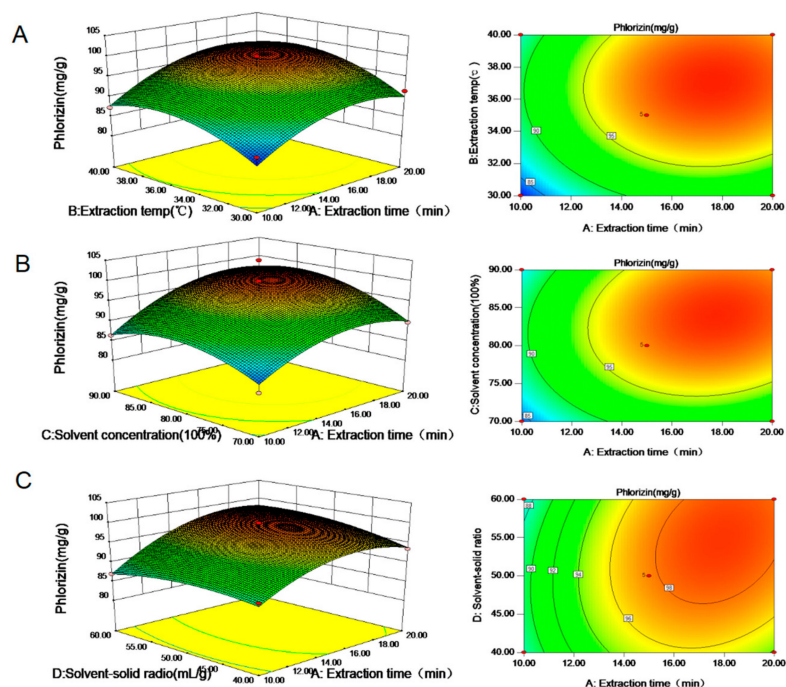


Figure 2. Cont.

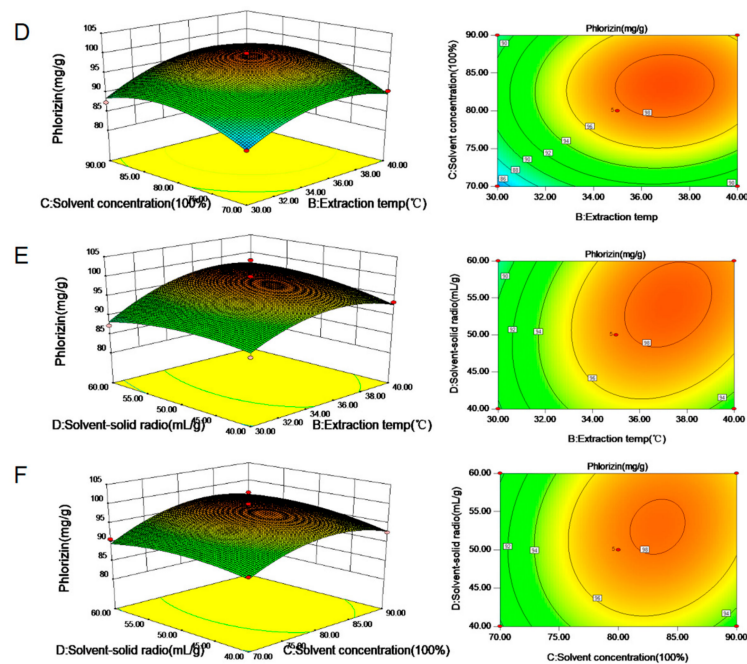


Figure 2. Response surface plots (3D) of phlorizin analysis as a function of significant interaction between factors. (A) Extraction temperature and extraction time. (B) Solvent concentration and extraction time. (C) Solvent-solid ratio and extraction time. (D) Solvent concentration and extraction temperature. (E) Solvent-solid ratio and extraction temperature. (F) Solvent-solid ratio and solvent concentration.

3.4. Determination of HUVEC Protection

To evaluate the cyto-protective activity of *M.toringoides* extracts, the maximal protection rate at different processing conditions was evaluated. The best protection rate obtained as result of UAE was 80.90% (18.60 min, 37.92 °C, 85.09%, and 57.47 mL/g).

As shown in Table 4, the model showed that X_1 , X_2 , X_3 , X_1^2 , X_2^2 , X_3^2 , and X_4^2 had significant effects on the results of oxidative stress in endothelial cells ($p < 0.05$). Based on variance analysis, the second term extraction time has the greatest impact on the protection rate. As shown in Figure 3a–c, cell viability increases with the increase of extraction time, reaching its maximum in about 15 min. Then a constant value is reached and it slowly decreases. Over time, the linear increase in the protective rate may be due to the continuous cavitation and mechanical stress resulting in the release of more active substances from plant tissues [30]. The correlation of the protection rate with the independent variables was demonstrated using the quadratic statistical equation below.

$$Y_1 = 50.05 + 4.99X_1 + 3.62X_2 + 3.06X_3 + 0.60X_4 + 0.61X_1X_2 + 1.09X_1X_3 + 1.48X_1X_4 + 0.70X_2X_3 + 1.42X_2X_4 + 0.70X_3X_4 - 4.87X_1^2 - 4.71X_2^2 - 4.80X_3^2 - 2.11X_4^2 \quad (2)$$

Table 4. Analysis of variance for the fitted quadratic polynomial model for protection rate.

Source	Sum of Squares	df	Mean Square	F-Value	p-Value, Prob > F	
Model	924.53	14	66.04	11.33	<0.0001	significant
X ₁ : Extraction time (min)	298.8	1	298.8	51.28	<0.0001	
X ₂ : Extraction temp (°C)	157.47	1	157.47	27.02	0.0001	
X ₃ : Solvent concentration (100%)	112.12	1	112.12	19.24	0.0006	
X ₄ : Solvent-solid ratio (mL/g)	4.36	1	4.36	0.75	0.4018	
X ₁ X ₂	1.5	1	1.5	0.26	0.6197	
X ₁ X ₃	4.75	1	4.75	0.82	0.3818	
X ₁ X ₄	8.79	1	8.79	1.51	0.2396	
X ₂ X ₃	1.96	1	1.96	0.34	0.5712	
X ₂ X ₄	8.07	1	8.07	1.38	0.259	
X ₃ X ₄	1.96	1	1.96	0.34	0.5712	
X ₁ ²	154.09	1	154.09	26.44	0.0001	
X ₂ ²	143.91	1	143.91	24.7	0.0002	
X ₃ ²	149.39	1	149.39	25.64	0.0002	
X ₄ ²	28.95	1	28.95	4.97	0.0427	
Residual	81.58	14	5.83			
Lack of Fit	54.96	10	5.5	0.83	0.6347	not significant
Pure Error	26.62	4	6.65			
Cor Total	1006.11	28				

In addition, the positive linear effect of extraction temperature and the negative effect of interaction between extraction temperature and solvent concentration are shown in Figure 3a,d,e. The linear positive correlation between cell viability and temperature may be due to higher system energy occurring at higher temperatures, which leads to more complete destruction of connective tissue and structural tissue, especially the internal force of binding and adhering analyte plant matrix molecules, which enhances the release and solubility of the E. Effective substance [31]. In addition, the extracts extracted at high temperature have higher protective activity, which is due to the increase of solvent diffusion and solubility of analytes. This is related to the antioxidant activity of solvents. The negative time/temperature interaction on the final product was observed at the higher end of the temperature axis of the surface graph, which indicates the adverse effect of long processing time (Figure 3a). As shown in Figure 3b,d,f, the higher protective rate associated with higher solvent concentration is due to increased solvent diffusion and enhanced solubility of biologically active chemicals in solvents. This helps obtain more effective substances. As shown in Figure 3c,e,f, when the liquid-solid ratio is 50 mL/g, the protection rate is higher than 40 mL/g. However, when the liquid-solid ratio is 60 mL/g, the protection rate is lower. The larger the liquid-solid ratio is, the larger the osmotic pressure and contact area are, the more solvents enter the plant matrix, and the more bioactive chemicals enter the solvents through the cell wall.

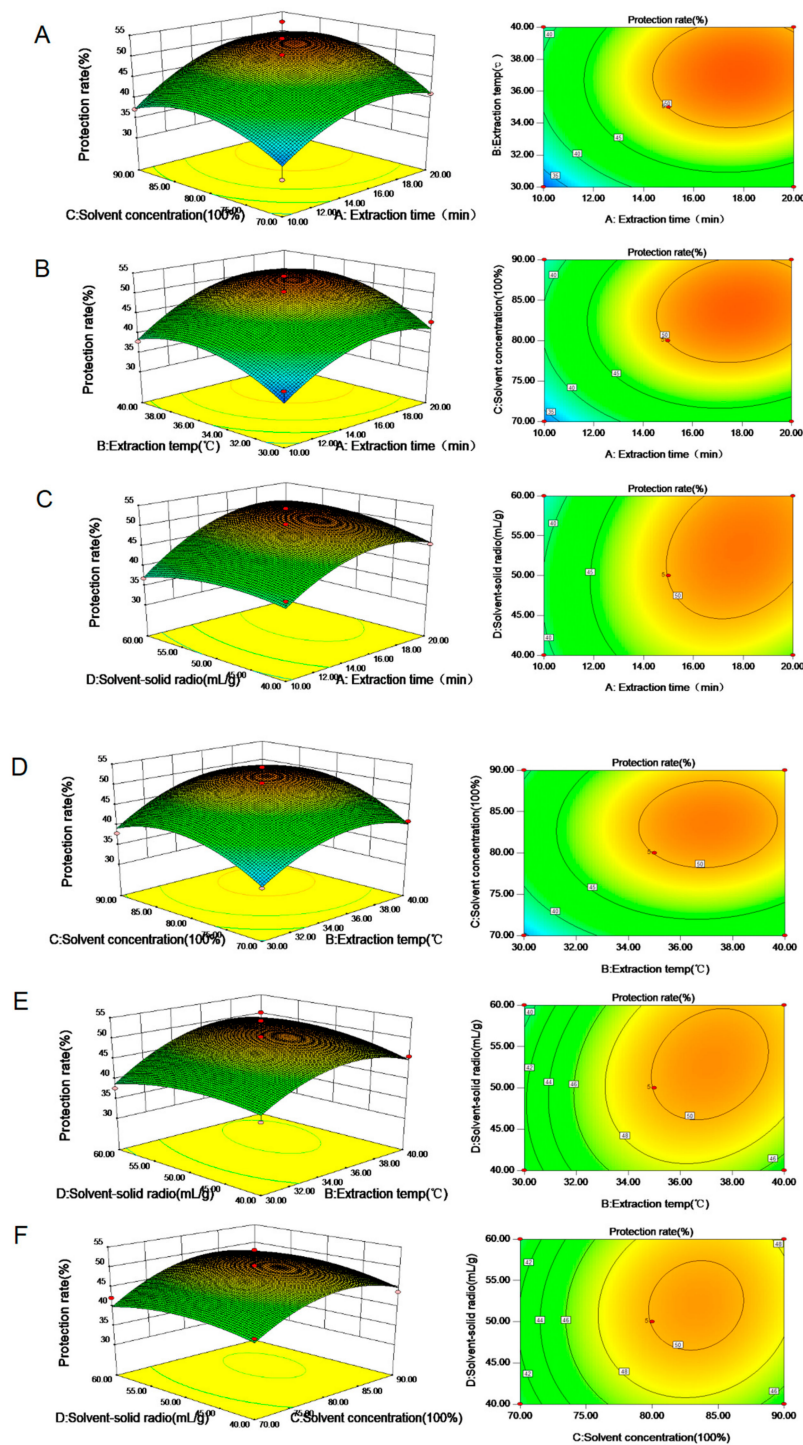


Figure 3. Response surface plots (3D) of protection rate analysis as a function of significant interaction between factors. (A) Extraction temperature and extraction time. (B) Solvent concentration and extraction time. (C) Solvent-solid ratio and extraction time. (D) Solvent concentration and extraction temperature. (E) Solvent-solid ratio and extraction temperature. (F) Solvent-solid ratio and solvent concentration.

3.5. Analysis of the Relationship between Protection Rate and Percentage of Phlorizin

To investigate the relationship between the rate of HUVEC protection against H_2O_2 -induced cell death and phlorizin content, 29 samples extracted by RSM were analyzed. Their relationship was revealed via a scatter plot, as shown in Figure 4.

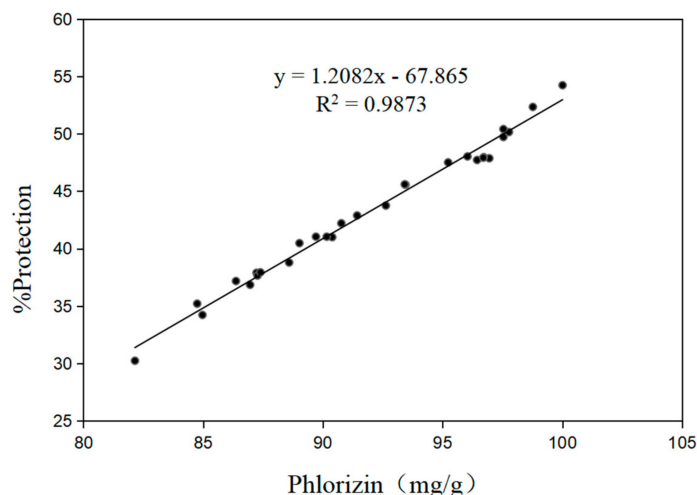


Figure 4. Scatter plot of the protection rate of human endothelial cells against hydrogen peroxide-induced oxidative stress versus phlorizin content of 29 *M.toringoides*.

We observed that the phlorizin content was highly correlated with the protection of HUVECs against oxidative stress, with an R^2 value of 0.9873, which suggests that this protection might be related to the phlorizin content in *M.toringoides* extracts.

4. Concluding Remarks

In the present study, optimization of ultrasound-assisted extraction to conserve the cyto-protective effect of *M.toringoides* was performed with a four-variable, three-level central composite design based on response surface methodology. Compared to conventional isolation, this novel approach is less tedious and less labor intensive. The optimal extraction parameters were determined to be a 58 mL/g liquid-solid ratio, a 38 °C extraction temperature, an 85% solvent concentration, and a 19-min extraction time. The protection rate against oxidative stress in human endothelial cells was $54.57 \pm 0.27\%$, which was similar to the predicted protection rate of 53.75%. The highest phlorizin content (101.239 mg/g) was also obtained using these conditions. We also show that phlorizin could be responsible for the cyto-protective effects of *M.toringoides* extracts.

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Conflicts of Interest: The authors declare no conflict of interest.

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