

# Optimization of Preparation Procedures of Phlorizin-Zn (II) Complex and Its Antioxidant Activity Study *in vitro*

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**Abstract:** Flavonoid metal complexes possess more effective antioxidant properties than flavonoids. However, the preparation of the phlorizin-Zn (II) complex and its antioxidant activity has not been reported. In this research, the preparation procedures of the phlorizin-Zn (II) complex were enhanced using by single factor test and Response Surface Methodology (RSM). The optimal preparation procedures of the phlorizin-Zn (II) complex were as follows: The time of 4.1 h, the pH of 5.9, the temperature of 48.7°C and the molar ratio (phlorizin: Zn<sup>2+</sup>) of 1:1. Under these conditions, the predicted preparation yield of phlorizin-Zn (II) complex was 79.052%. Additionally, the antioxidant activity of the phlorizin-Zn (II) complex was analyzed in this research. 1, 1-Diphenyl-2-Picrylhydrazyl (DPPH), 2, 2'-Azino-bis (3-Ethylbenzothiazoline-6-Sulfonic acid) (ABTS), hydroxyl free radical and Ferric ion Reducing Antioxidant Power (FRAP) experiments revealed that phlorizin-Zn (II) complex had significant DPPH, ABTS and hydroxyl free radical scavenging abilities and exhibited substantial reducing power in a dose-dependent manner. Moreover, the phlorizin-Zn (II) complex displayed stronger *in vitro* antioxidant capacity compared with phlorizin. The findings demonstrated that the phlorizin-Zn (II) complex had great potential in antioxidant applications in the future.

**Keywords:** Phlorizin-Zn (II) Complex, Preparation Procedure, Response Surface Methodology, Antioxidant Activity

## Introduction

Polyphenolic secondary metabolic substances in plants are known as flavonoids (Khalid *et al.*, 2019). There are several biological and pharmacological characteristics displayed by flavonoids, including neuroprotective, anti-inflammatory, anti-cancer, antibacterial, and antidiabetic properties (Ullah *et al.*, 2020). Phlorizin is one of the most common flavonoids found in apples which is plentiful in both the unpeeled and peeled parts (Gosch *et al.*, 2010). Phlorizin has several pharmacological actions, such as hypoglycemic, antioxidant, and anti-inflammatory properties (Jia *et al.*, 2021). It was discovered that the phlorizin from lotus seed epicarp had high FRAP values and was effective in scavenging DPPH and ABTS radicals (Ma *et al.*, 2019). Previous research showed that phlorizin increased the activity of antioxidant enzymes in *Drosophila* and prolong the life span, improve the survival ability, and reduce the mortality of *Drosophila* treated with paraquat and H<sub>2</sub>O<sub>2</sub> (Wang *et al.*, 2019). Therefore, phlorizin shows great potential in antioxidant activity.

After the formation of complexes between flavonoids and metal ions, their antibacterial, anti-inflammatory, anticancer, hypoglycemic, and antioxidant biological activities will be significantly enhanced (Kisku *et al.*, 2022; Li *et al.*, 2022; Malacaria *et al.*, 2022; Raza *et al.*, 2016; Yang *et al.*, 2014). The potent chelating ability of flavonoids with several metal ions, including Zn (II), Cu (II), Fe (II), Cr (III), Co (II), Mn (II), and Pb (II), has been studied extensively. Jayaprakash *et al.* (2023); Kejik *et al.* (2021) Zinc is a trace element that is necessary for human health. It is crucial for growth and development, immunological response, brain function, reproduction, etc., (Cummings and Kovacic, 2009). The findings showed that the newly created compound Rutin-zinc (II) was a more effective antioxidant *in vitro* and displayed more significant anti-tumor ability on leukemia, multiple myeloma, and melanoma cell lines than free rutin (Ikeda *et al.*, 2015). Another study revealed that the radical scavenging effect of the Iso-orientin-Zinc complex was substantially greater than that of iso-orientin (Wang *et al.*, 2021). However, the preparation and antioxidant activity of the phlorizin-Zn (II) complex remains unclear.

In this study, single-factor testing and RSM were used to improve the phlorizin-Zn (II) complex preparation procedure, and the optimal preparation process with the best preparation rate was obtained. The antioxidant activity of the phlorizin-Zn (II) complex was determined using DPPH, ABTS, hydroxyl free radical scavenging, and FRAP test. This study will provide a deeper understanding of the functions of the phlorizin-Zn (II) complex and lay the groundwork for its future development and utilization.

## Materials and Methods

### Materials

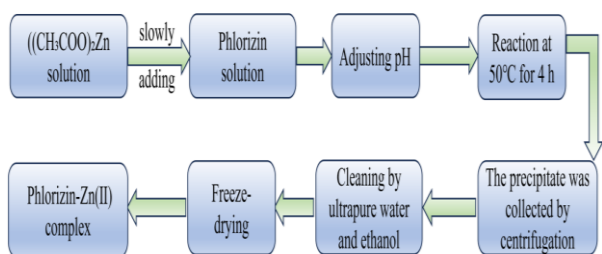
Phlorizin ( $\geq 98\%$ ), DPPH, and ABTS were obtained from Yuanye Biotechnology Co., Ltd (Shanghai, China). Salicylic acid was purchased from Sigma-Aldrich (St Louis, MO, USA). More chemicals were ordered from Sinopharm Chemical Reagent Co., Ltd (Beijing, China) and were of analytical grade. Ultrapure water was prepared for the experiment.

### Preparation of Phlorizin-Zn (II) Complex

The zinc acetate ( $(\text{CH}_3\text{COO})_2\text{Zn}$ ) solution (0.1 mol/L) was gradually incorporated into phlorizin solution (0.1 mol/L) to achieve a molar ratio of 1:1 and the pH was adjusted to 6 by sodium acetate solution. The precipitate was collected by centrifugation following a 4 h reaction at  $50^\circ\text{C}$  and the ultrapure water and ethanol were then cleaned, respectively. Phlorizin-Zn (II) complex was obtained by freeze-drying. The preparation flow chart of the phlorizin-Zn (II) complex is shown in Fig. 1.

### Single Factor Experimental Design

To examine the impact of several variables on the yield of phlorizin-Zn (II) complex, single-factor experiments were used. pHs were set at 5-9. Reaction temperatures were set at  $30\text{-}70^\circ\text{C}$ . Reaction times were set at 1-5 h and the molar ratios of phlorizin to metal ions were set at 1:1, 1:2, 1:3, 2:1, and 3:1.



**Fig. 1:** The preparation flow chart of phlorizin-Zn (II) complex

**Table 1:** The range of independent parameters in the design of the experiment on the phlorizin-Zn(II) complex

	Levels		
	-1	0	+1
Time ( $X_1$ )/h	3	4	5
pH ( $X_2$ )	5	6	7
Temperature ( $X_3$ )/ $^\circ\text{C}$	40	50	60

### Box-Behnken Design (BBD) for Phlorizin-Zn (II) Complex

A three-factor, three-level response surface analysis experiment was designed based on three factors that significantly affected the yield of the phlorizin-Zn (II) complex. The yield of the phlorizin-Zn (II) complex was taken as the response value (Sun *et al.*, 2023). The amounts of coded and uncoded independent variables are shown in Table 1. A total of 17 experimental runs in random order were needed. The quadratic regression model was stated in its general form as follows (Kadam *et al.*, 2023):

$$Y = \beta_0 + \sum_{i=1}^3 \beta_i X_i + \sum_{i=1}^3 \beta_{ii} X_i^2 + \sum_{i < j} \sum_{i=1}^3 \beta_{ij} X_i X_j \quad (1)$$

where,  $Y$  is the predicted phlorizin-Zn (II) complexed yield;  $\beta_0$  is the constant term.  $\beta_i$  is the regression coefficient of the linear term,  $\beta_{ii}$  is the regression coefficient of the quadratic term and  $\beta_{ij}$  is the regression coefficient of the interaction term.  $X_i$  and  $X_j$  are the coded independent variables.

### DPPH Free Radical Scavenging Assay

DPPH solution of  $100 \mu\text{mol/L}$  was prepared with anhydrous ethanol as solvent and stored in the dark. The phlorizin or phlorizin-Zn (II) complex solution (final concentration  $0.5\text{-}5 \text{ mg/mL}$ ) of the same volume was mixed with DPPH solution and the reaction was completed at  $298 \text{ K}$  in a dark state for 30 min. Every group's absorbance was calculated when the wavelength was  $517 \text{ nm}$  (Xiao *et al.*, 2020). The DPPH radical scavenging ability of phlorizin and phlorizin-Zn (II) complex was determined using the following equation:

$$\text{DPPH radical scavenging rate (\%)} = \left( 1 - \frac{A_1 - A_2}{A_3} \right) \times 100\% \quad (2)$$

where,  $A_1$ ,  $A_2$  and  $A_3$  were the absorbance of the sample and DPPH solution, the sample without the DPPH solution, and the ethanol and DPPH solution, respectively.

### ABTS Free Radical Scavenging Assay

ABTS solution (10 mL; 7 mmol/L) was thoroughly mixed with  $K_2S_2O_8$  solution (5 mL; 7.35 mmol/L) and then the mixed solution was stored under light-free conditions for 16 h to obtain the ABTS stock solution. Then ABTS reserve solution was prepared by dilution with anhydrous ethanol and detected at 734 nm until the absorbance value of the ABTS working solution was  $0.7 \pm 0.02$ . ABTS working solution (1 mL) was combined with different concentrations of phlorizin or phlorizin-Zn (II) complex (200  $\mu$ L; final concentration 0.05-0.5 mg/mL) and the mixed solution was placed in the dark at room temperature for 6 min. Every group's absorbance was calculated when the wavelength was 734 nm (Rumpf *et al.*, 2023). The following formula was used to determine the ABTS radicals' scavenging activity:

$$\text{ABTS radical scavenging ability (\%)} = \left(1 - \frac{A_1}{A_2}\right) \times 100 \quad (3)$$

where,  $A_1$  is the absorbance of the ABTS working solution and different concentrations of samples.  $A_2$  is the absorbance of distilled water mixed with ABTS working solution.

### Hydroxyl Radical Scavenging Assay

The different concentration of phlorizin or phlorizin-Zn (II) complex solution (100  $\mu$ L) was mixed with  $Fe_2SO_4$  solution (200  $\mu$ L, 1.8 mmol/L) and salicylic acid-ethanol solution (1 mL, 1.8 mmol/L) then  $H_2O_2$  solution (10  $\mu$ L, 0.03%) was added to the mixture. The mixture was kept at 310 K for 30 min. The absorbance value was measured at 510 nm wavelength (Sihag *et al.*, 2022). The following formula was used:

$$\text{OH radical scavenging rate (\%)} = \left(1 - \frac{A_1 - A_2}{A_0}\right) \times 100\% \quad (4)$$

where,  $A_1$  is the absorbance of the mixture with the sample,  $Fe_2SO_4$  solution, salicylic acid-ethanol solution, and  $H_2O_2$  solution.  $A_2$  is the absorbance of the mixture with sample and ultrapure water.  $A_3$  is the absorbance of the mixture of ultrapure water,  $Fe_2SO_4$  solution, salicylic acid-ethanol solution, and  $H_2O_2$  solution.

### FRAP Assay

Acetate buffer (100 mL; pH 3.5), TPTZ solution (2,4,6-tri (2-pyridyl)-s-triazine; 10 mL; 10 mmol/L) and ferric chloride solution (10 mL; 20 mmol/L) were mixed and placed at 310 K for 1 h to prepare the FRAP solution. The preheated FRAP solution (150  $\mu$ L) was mixed evenly

with ultrapure water (15  $\mu$ L) and the phlorizin and phlorizin-Zn (II) complex solution (5  $\mu$ L; final concentration 0.05-0.5 mg/mL). The mixed solution was then left at a temperature of 310 K for 30 min in the absence of light to allow for full reaction. The absorbance of the mixture was measured at 593 nm. Every group's absorbance was calculated when the sample solution was replaced with  $FeSO_4$  standard solution of different concentrations and the operation was repeated according to the above steps to draw the standard curve (Wootton-Beard *et al.*, 2011).

### Statistical Analysis

Analysis of Variance (ANOVA) was used to examine the data, which were reported as mean  $\pm$  Standard Deviation (SD). Design expert software (10.0.7) was used for experimental design and statistical analysis.

## Results and Discussion

### Single Factor Experiment for the Preparation of Phlorizin-Zn (II) Complex

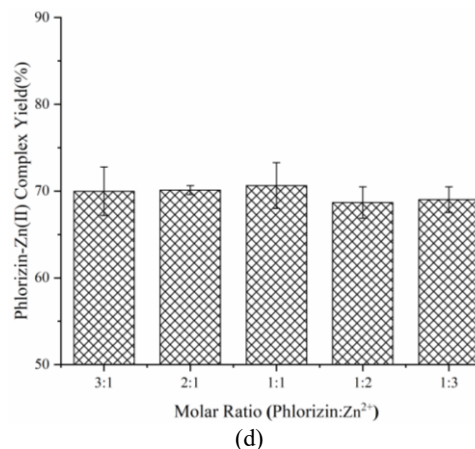
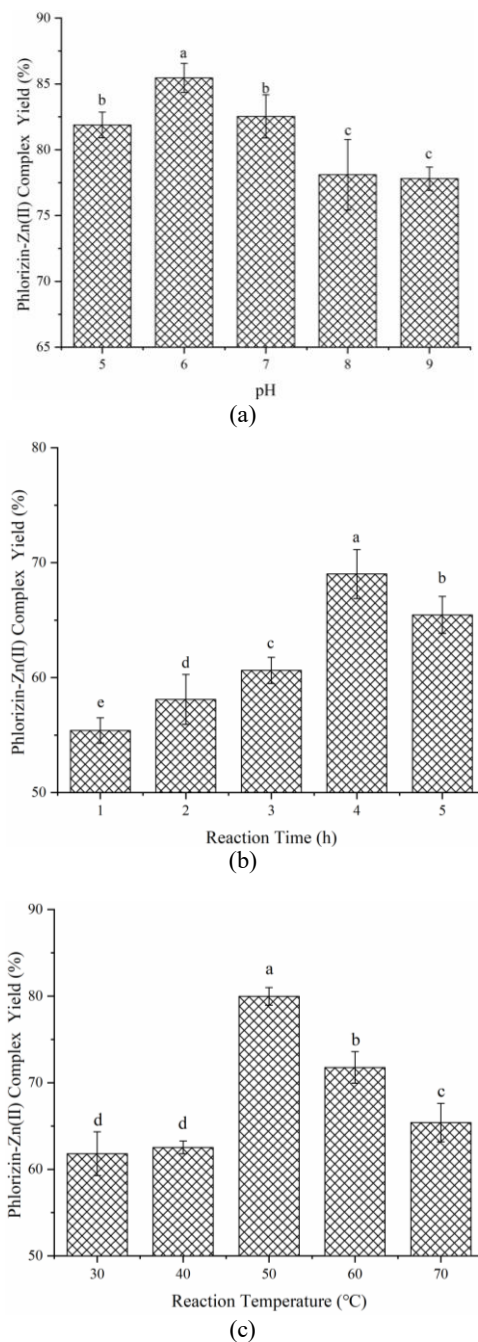
#### Effect of Reaction pH on the Yield of Phlorizin-Zn (II) Complex

The acid-base property of reaction solution has great influence on the degree of metal dissociation (An *et al.*, 2022). Therefore, the pH value has a great influence on the coordination reaction during the test. The yields of phlorizin-Zn (II) complex at different pH were measured and shown in Fig. 2(a). When the pH increased, the yield of phlorizin-Zn (II) complex increased continuously, however when the pH was greater than 6, the yield of phlorizin-Zn (II) complex began to decrease. Meanwhile the yield of the phlorizin-Zn (II) complex under acidic conditions was higher than under alkaline conditions. The possible cause was that metal ions were more easily dissociated under acidic conditions, which was conducive to the progress of the reaction. The highest yield of the phlorizin-Zn (II) complex reached 85.25% when the pH of the reaction solution was 6. Other study revealed that the changes of pH affected the formation of tea polysaccharide (TPS) complexes and TPS complexes were more easily formed when the reaction pH was close to the pH of the TPS solution (Fan *et al.*, 2021).

#### Effect of Reaction Time on the Yield of Phlorizin-Zn (II) Complex

The reaction time of flavonoids and metal ions is directly related to whether the coordination reaction is complete. Fig. 2(b) showed that the yields of phlorizin-Zn(II) complex at different reaction time. The yield of phlorizin-Zn (II) complex progressively increased and then decreased as the extension of reaction time and the

yield got the max (79.01%) at 4 h. The reason might be that when the reaction time was short, the reaction of zinc acetate and phlorizin was insufficient. Then when a generation of the complex reached saturation in the reaction after a long time, the reverse reaction was greater than the forward reaction, which resulted in a decrease in the yield of the phlorizin-Zn (II) complex. In other research, the reaction time in the preparation of gallic acid metal complex was 3 h (El-Megharbel and Hamza, 2022). It could be seen that different flavonoids-metal complex had different preparation times.



**Fig. 2:** The effect of pH (a); Reaction time (b); Reaction temperature (c); and Molar ratio (d); On phlorizin-Zn complex yield. Different letters indicate significant differences ( $p < 0.05$ )

### Effect of Reaction Temperature on the Yield of Phlorizin-Zn (II) Complex

Temperature has an important impact on the rate of the chemical reaction (Eliason and McMahon, 1981). The rise of temperature often encourages the coordination reaction between flavonoids and metal ions. The yields of phlorizin-Zn (II) complex at different reaction temperatures were measured as Fig. 2(c) illustrates. As the temperature raised the yield of the phlorizin-Zn (II) complex increased. The yield of the phlorizin-Zn (II) complex reached the maximum of 79.98% at 50°C. However, with a further increase in temperature, the yield of the phlorizin-Zn (II) complex began to decrease. The 55°C was selected as the reaction temperature when prepared the iso-orientin-metal complex, which was similar to our research (Wang *et al.*, 2021). The explanation might be that as the temperature rose, the chelating ability of phlorizin and zinc ions continued to increase, resulting in the continuous increase of yield of the phlorizin-Zn (II) complex. However, with the further increase in temperature, the thermal stability of phlorizin was affected and the molecular structure of phlorizin was destroyed by long heat treatment.

### Effect of the Molar Ratio on the Yield of Phlorizin-Zn (II) Complex

Figure 2(d) illustrates that the influence of the molar ratio on the yield of the phlorizin-Zn (II) complex. The yields of phlorizin-Zn (II) complex were no significant difference in the dosage ratio of different molar ratios. So, we chose the molar ratio (phlorizin: Zn<sup>2+</sup>) of 1:1 for follow-up experiments.

**Table 2:** Optimization of preparation parameters for phlorizin-Zn(II) complex by response surface design

Run	X <sub>1</sub> (h)	X <sub>2</sub>	X <sub>3</sub> (°C)	Y (%)
1	3	6	60	65.49
2	3	6	40	68.32
3	3	5	50	71.50
4	4	6	50	78.46
5	4	6	50	80.45
6	5	7	50	74.58
7	4	6	50	79.50
8	3	7	50	69.35
9	4	5	40	75.32
10	4	6	50	77.85
11	4	6	50	78.26
12	4	7	60	71.43
13	4	5	60	70.25
14	5	6	60	68.10
15	4	7	40	71.85
16	5	6	40	70.32
17	5	5	50	71.21

### Optimization of the Preparation Process by RSM

#### Experimental Design and Result Analysis by RSM

Based on the single-factor test, pH, time, and temperature were taken as test factors to conduct RSM. Table 2 lists the three components, their levels, and the RSM results. Multiple regression analysis using the experimental data obtained, the quadratic regression equation of the actual values of the factors in this model was obtained. The relationship between preparation yield and the independent variable was verified by a mathematical model. The final equation based on the coding factor was as follows:

$$Y = 78.90 + 1.19375X_1 - 0.1337X_2 - 1.317X_3 + 1038X_1X_2 + 0.152X_1X_3 + 1.1625X_2X_3 - 5.699X_1^2 - 1.5445X_2^2 - 5.147X_3^2 \quad (5)$$

where, Y is the yield of the phlorizin-Zn (II) complex. X<sub>1</sub> is the coded variable for time, X<sub>2</sub> is the coded variable for PH and X<sub>3</sub> is the coded variable for temperature.

Table 3 presented the results of the experimental analysis of variance, the model (p<0.0001) was sky-high significant. According to statistics, the model's misfit was not significant (p>0.05). The trial's predicted and actual values were determined to be well-matched by the R<sup>2</sup> value of 0.9815. The adjusted coefficient of determination (R<sup>2</sup><sub>adj</sub> = 0.9576) and the prediction coefficient of determination (R<sup>2</sup><sub>pred</sub> = 0.9012) were similar (R<sup>2</sup><sub>adj</sub> - R<sup>2</sup><sub>pred</sub> < 0.2), indicating that this regression equation was reliable and could accurately reflect the relationship of phlorizin-Zn (II) complex yield between response values and various experimental factors (Akalin *et al.*, 2015; Cheng *et al.*, 2020; Hao *et al.*, 2023).

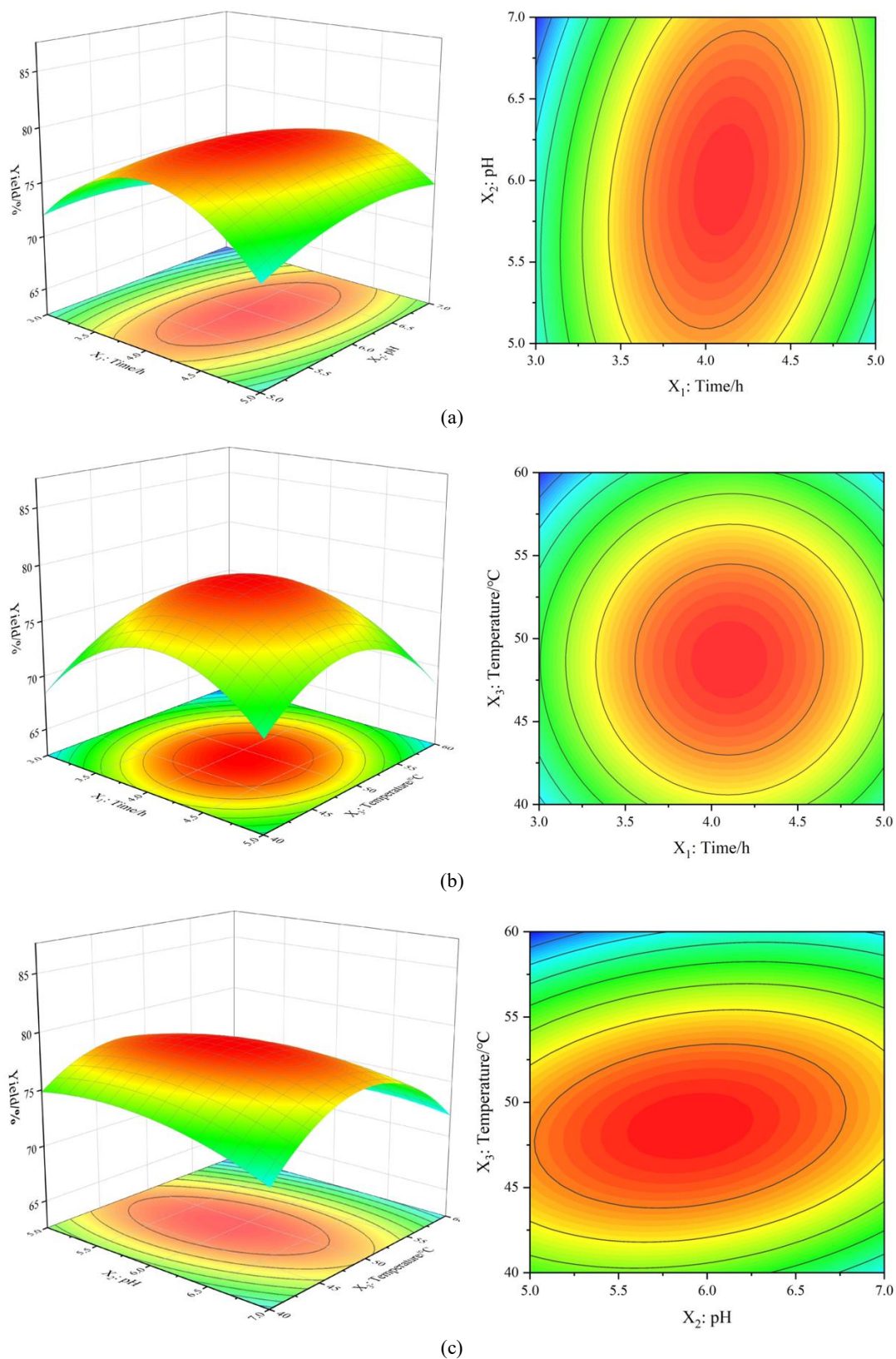
The F-value and p-value of each model term were used as tools to evaluate each parameter's importance. The magnitude of the F-value was proportional to the effect of this factor on the response surface (Hatami *et al.*, 2023; Latif *et al.*, 2022). Table 3 demonstrated that the yield of the phlorizin-Zn (II) complex was significantly influenced (p<0.05) by linear coefficients (X<sub>1</sub>, X<sub>3</sub>), cross coefficients (X<sub>1</sub>X<sub>2</sub>, X<sub>2</sub>X<sub>3</sub>), and quadratic coefficients (x<sub>1</sub><sup>2</sup>, x<sub>2</sub><sup>2</sup>, x<sub>3</sub><sup>2</sup>). While there was no significant coefficient of other terms (p>0.05). Therefore, the reaction temperature (X<sub>3</sub>) and reaction time (X<sub>2</sub>) followed by pH (X<sub>1</sub>) were the main factors affecting the phlorizin-Zn (II) complex yield.

**Table 3:** Regression coefficients and ANOVA estimated for phlorizin-Zn(II) complex yield

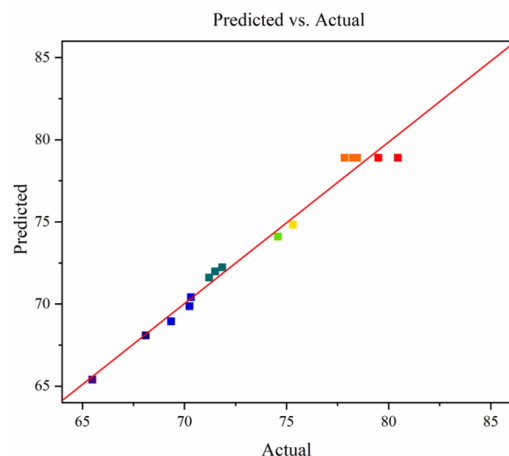
Source	Sum of squares	Df	Mean square	F-value	p-value	
Model	320.012	09	35.057	41.18	<0.0001	**
X <sub>1</sub>	11.040	01	11.040	13.20	0.0084	**
X <sub>2</sub>	0.014	01	0.014	0.17	0.6961	ns
X <sub>3</sub>	13.089	01	13.089	16.08	0.0051	**
X <sub>1</sub> X <sub>2</sub>	7.062	01	7.062	8.82	0.0208	*
X <sub>1</sub> X <sub>3</sub>	0.093	01	0.093	0.11	0.7524	ns
X <sub>2</sub> X <sub>3</sub>	5.041	01	5.041	6.26	0.0409	*
X <sub>1</sub> <sup>2</sup>	136.078	01	136.078	158.36	<0.0001	**
X <sub>2</sub> <sup>2</sup>	10.004	01	10.004	11.63	0.0113	*
X <sub>3</sub> <sup>2</sup>	111.054	01	111.054	129.15	<0.0001	**
Residual	6.005	07	0.086			
Lack of Fit	1.058	03	0.053	0.47	0.7189	ns
Pure Error	4.047	04	1.012			
Cor Total	326.016	16				
R <sup>2</sup> = 0.9815						
R <sup>2</sup> <sub>adj</sub> = 0.9576						
R <sup>2</sup> <sub>pred</sub> = 0.9012						

\*, the difference is significant at 0.05 level (p<0.05); \*\*, the difference is significant at 0.01 level (p<0.01); ns, the difference is not significant (p>0.05)





**Fig. 3:** Response surface plots and contour plots for interaction between various factors on the yield of phlorizin-Zn (II) complex, time and pH (a); Time and temperature (b); and pH and temperature (c)



**Fig. 4:** Linear regression plots of the predicted and experimental values for phlorizin-Zn (II) complex yield

#### *Analysis of Response Surface Plot and Contour Plot*

The contour plot's contour line's shape signifies the degree to which the two test elements interact. An evident interaction between the two test factors is shown by the ellipse contour line, whilst a not-obvious interaction is indicated by the circle contour line. The higher the density of the contour line, the more significant the impact of the experimental factor on the response value. In the 3D response surface plot, the steeper the surface made the impact of experimental variables on the response value more significant (Alsawalha, 2023; Jang *et al.*, 2017; Obregón *et al.*, 2023; Yuan *et al.*, 2022).

As can be seen from Fig. 3(a), when the temperature ( $X_3$ ) was at the level of 0, the yield continued to increase with the increase of time ( $X_1$ ) and pH ( $X_2$ ). After obtaining the highest yield of phlorizin-Zn (II) complex related to pH-time, inappropriate pH and time lead to a decrease in yield. Figure 3(b) evicted the interaction of time ( $X_1$ ) and temperature ( $X_3$ ) on the preparation yield of the phlorizin-Zn (II) complex. As temperature and time increased, the yield rose as well. When the yield reached the maximum value, there was no further increase. However, the contour shape tended to be circular, indicating that the interaction between time and temperature was not obvious. When the time ( $X_1$ ) was fixed at the level of 0, the effect of pH ( $X_2$ ) and temperature ( $X_3$ ) interaction on the preparation yield of phlorizin-Zn (II) complex in the contour plot and 3D response plot was shown in Fig. 3(c). When pH and temperature was 6 and 50, respectively, the preparation yield of phlorizin-Zn (II) complex was the highest. The preparation yield of the phlorizin-Zn (II) complex declined when the pH and temperature were increased further.

#### *Verification of Predictive Model*

An ideal method was used to generate a response surface design experimental model to maximize yield by

optimizing independent parameters such as pH, temperature, and time. Figure 4 demonstrated that there was no discernible difference between the predicted and actual values. The optimal preparation scheme of the phlorizin-Zn (II) complex was obtained by design expert software analysis as follows: The time of 4.097 h, the pH of 5.951, the temperature of 48.678°C and the molar ratio (phlorizin:  $Zn^{2+}$ ) of 1:1. Under these conditions, the predicted preparation yield of phlorizin-Zn (II) complex was 79.052%. Considering the feasibility of actual operation, the optimal conditions given by the model were rounded, so the optimal preparation conditions included. The time of 4.1 h, the pH of 5.9, the temperature of 48.7°C, and the molar ratio (phlorizin:  $Zn^{2+}$ ) of 1:1. Under these experimental circumstances, the verification test was conducted and the average preparation yield was determined to be 78.02% through three parallel determinations, which quite closed to the predicted value. The outcomes demonstrated the model's dependability and suitability for use as a guide in determining the precise process parameters.

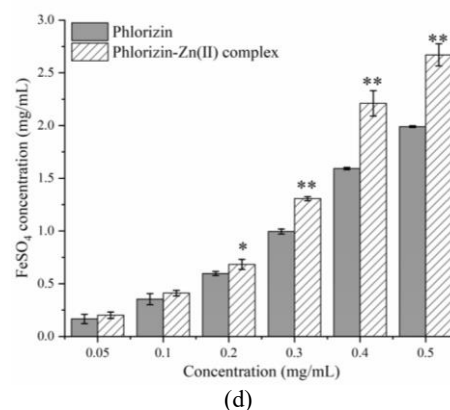
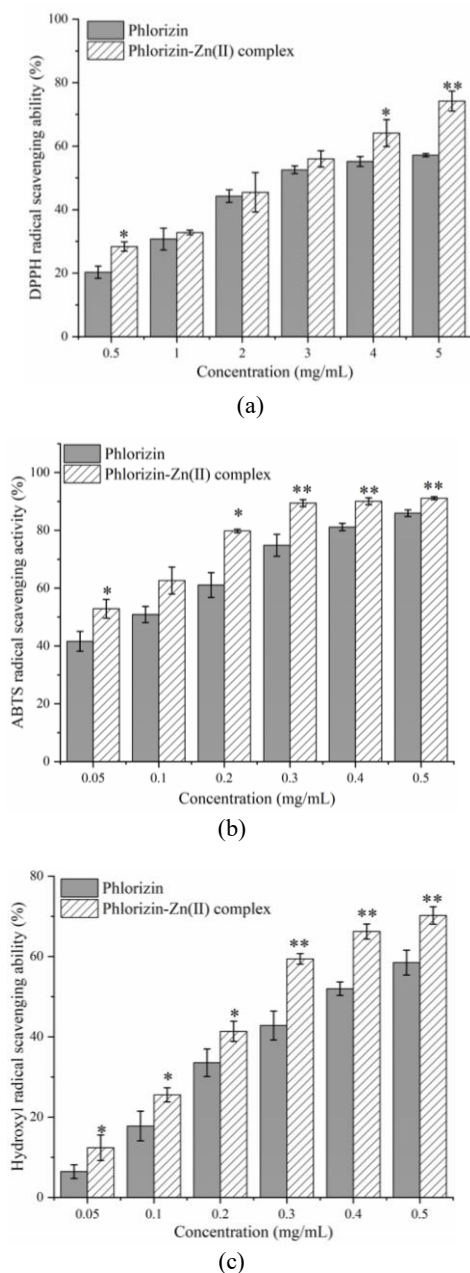
#### *The Antioxidative Activity of Phlorizin-Zn (II) Complex DPPH Free Radical Scavenging Activity*

Determining the DPPH free radical scavenging capacity is a typical way to assess the antioxidant potential of natural products and bioactive substances since DPPH is a free radical that is quite stable (Alamgir *et al.*, 2014). Figure 5(a), the DPPH free radical scavenging rates of phlorizin and phlorizin-Zn (II) complex were dose-dependent within the concentration range of 0.5-5 mg/mL. When the dosage was 5 mg/mL, the DPPH free radical scavenging rates of phlorizin and phlorizin-Zn (II) complex were 57.16 and 74.21% respectively. Thus, The DPPH scavenging ability of the phlorizin-Zn (II) complex was higher than that of phlorizin. Quercetin-iron complexes were substantially more effective in scavenging DPPH radicals than free quercetin. This demonstrates that quercetin-iron complexes were far stronger antioxidants than quercetin (Raza *et al.*, 2016). Flavone's potent antioxidant qualities are attributed to its many binding sites. In addition, metal ions also have antioxidant activity (Van Acker *et al.*, 1996). Therefore, by chelating metal ions, flavonoid metal complexes could better prevent the production of free radicals and their subsequent reactions to improve antioxidant activity (Wang *et al.*, 2021).

#### *ABTS Free Radical Scavenging Activity*

The greatest absorption peak of the blue-green ABTS<sup>+</sup> free radical solution was 734 nm. When the interaction occurs, the color of the ABTS<sup>+</sup> radical solution gradually becomes lighter and when the phenomenon of color change exists, it represents the ability to have antioxidant activity (Marc *et al.*, 2004). In a dose-dependent manner, phlorizin and phlorizin-Zn (II) complex scavenged ABTS free radicals (Fig. 5(b)).

When the dosage was 0.05-0.5 mg/mL, the ABTS scavenging rate of phlorizin-Zn (II) complex was stronger than that of phlorizin. When the dosage was 0.4 mg/mL, the ABTS free radical scavenging capacity of the phlorizin-Zn (II) complex was 11% higher than that of phlorizin ( $p < 0.05$ ). Flavonoid metal complexes maintain all flavonoid properties and their pharmacokinetic characteristics are enhanced after being combined with metal ions (Kasprzak *et al.*, 2015). It was discovered that following the coordination process between metal ions and flavonoids, hyper-delocalized structures with more active sites were formed and these active sites further improved the metal-chelated flavonoids' capacity to scavenge free radicals (Zhang *et al.*, 2020).



**Fig. 5:** Inhibition effects of phlorizin-Zn (II) complex on DPPH radicals (a); ABTS radicals (b); Hydroxyl radicals (c); and reducing power (d); \* $p < 0.05$ , \*\* $p < 0.01$  vs phlorizin group

### Hydroxyl Radical Scavenging Activity

The most hazardous and poisonous active oxygen-free radical that organisms are aware of is hydroxyl (Yu *et al.*, 2021). High reactive hydroxyl free radical has strong oxidation on potassium chromate and potassium permanganate and was the three-electron reduction product of oxygen. It interacts with a variety of molecules in the biological body through hydrogen extraction, addition, or electron transfer, causing oxidation and damage to proteins, amino acids, nucleic acids, and lipids, finally resulting in cell necrosis or mutation (Purushothaman *et al.*, 2020). Hydroxyl radicals were also associated with tumor, phagocytosis, senescence, and radiation damage (Rafat Husain *et al.*, 1987). Figure 5(c) showed that both phlorizin and phlorizin-Zn (II) complex had a strong scavenging effect on hydroxyl radicals and the scavenging abilities were proportional to their concentrations. However, the scavenging activity of phlorizin was lower than that of the phlorizin-Zn (II) complex at the same concentration. The highest scavenging rate of the phlorizin-Zn (II) complex reached 70.22% and its IC<sub>50</sub> was 0.293 mg/mL. The stronger hydroxyl free radical scavenging ability of phlorizin-Zn (II) complex than free phlorizin might be ascribed to the synergistic effect of phlorizin and Zn<sup>2+</sup> which promoted the contact between phlorizin-Zn (II) complex and hydroxyl free radical and then improved the antioxidant property of phlorizin-Zn (II) complex (Zhang *et al.*, 2020). Previous reports also found that the Iso-orientin-Zinc complex had a greater hydroxyl radical scavenging capacity than iso-orientin in a certain concentration range (Wang *et al.*, 2021).

### FRAP Assay

In the FRAP Assay, the total antioxidant substance in the sample reduces Fe<sup>3+</sup> to Fe<sup>2+</sup>, then Fe<sup>2+</sup> and tripyridyl-triazine form a blue-purple complex with strong absorption at 593 nm wavelength. Thus FRAP assay not



only reflects the scavenging activity of a certain free radical but also reflects the total antioxidant capacity of the sample (Bolanos De *et al.*, 2015; Jones *et al.*, 2017). The higher FeSO<sub>4</sub> concentration in the reaction mixture indicates the more significant reduction capacity of antioxidants. Figure 5(d), at the 0.05-0.5 mg/mL concentration range, the FeSO<sub>4</sub> concentration increased gradually with the increase of phlorizin and phlorizin-Zn (II) complex concentration, indicating that the FRAP activities of phlorizin and phlorizin-Zn (II) complex were gradually enhanced. At the same concentration, the FRAP activity of the phlorizin-Zn (II) complex was better than phlorizin. When the concentration was 0.5 mg/mL, the FRAP activity of the phlorizin-Zn (II) complex was 23.9% which was higher than that of phlorizin. The results demonstrated the electron-donating property of the phlorizin-Zn (II) complex for neutralizing free radicals and the formation of stable products. Another study also showed that the ability of the metal curcumin complex to reduce iron ions is stronger than free curcumin (Joshi *et al.*, 2023). In summary, the FRAP of the phlorizin-Zn (II) complex was stronger than that of free phlorizin.

## Conclusion

In this study, the preparation technology of the phlorizin-Zn (II) complex was optimized. The results showed that the maximum yield of phlorizin-Zn (II) complex was 78.02%, which was quite close to the predicted value, while the parameters at this time were 4.1 h, pH 5.9, temperature 48.7°C and the molar ratio (phlorizin: Zn<sup>2+</sup>) was 1:1, respectively. Moreover, the phlorizin-Zn (II) complex had stronger free radical scavenging ability and reducing power than phlorizin. The synergistic effect of phlorizin and Zn<sup>2+</sup> improved the antioxidant property of the phlorizin-Zn (II) complex. More importantly, future work will focus on the mechanism of its functional action.

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## Author's Contributions

**Yuqing Zhang:** Participate in the whole experimental designed, experimental method, result analysis and manuscript written.

**Yaping Li:** Participate in part of the experimental process and the analysis of results.

**Guorui Yang:** Participated in part of the experiment.

**Shaoxuan Yu:** Ameliorated the manuscript.

**Haifang Xiao:** Participated in the experiment designed guidance, result interpretation, and ameliorated the manuscript.

**Yuanda Song:** Participated in part of the guidance of experimental designed and ameliorated the manuscript.

## Ethics

The original content in this piece hasn't been released before. The corresponding author attests that there are no ethical concerns and that every other author has read and approved the paper.

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